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α-Amylase from *Bacillus subtilis*: III. Effects of Inhibitors upon Liquefaction and Dextrinization

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Blom, Bak and Braae (1) reduced the activity of pancreatic, bacterial (Superclastase), and certain *Aspergillus* amylase preparations by heat, acid, or adsorption on starch granules. The parallel changes which were observed in the reducing, liquefying and dextrinizing powers of these enzyme preparations led to the conclusion that a single enzyme (α-amylase) was functioning. Hollenbeck and Blish (2) subjected bacterial amylase, takadiastase, malt α-amylase, and a malt extract to partial inactivation by heat and by pH changes. Determinations of the remaining liquefying and dextrinizing activities indicated losses in each to approximately the same extent. This correlation indicated that α-amylase was responsible for both manifestations of starch hydrolysis.

Despite these findings, volumes of recent publication (3, 4) persist in the presentation of the view that liquefaction and dextrinization result from the hydrolytic cleavage of starch by a liquefying amylase and by α-amylase, respectively.

The denaturation and adsorption investigations (1, 2, 5) show that liquefaction and dextrinization are functions of a single enzyme. This if further substantiated by our work (6), in which it was found that the ratio of liquefying activity to dextrinizing activity of bacterial amylase remained constant after purification which effected a 900-fold increase in potency. These findings, however, do not preclude the rather remote possibility of this molecule containing different groups essential to each process. It has been demonstrated that α-amylase from *B. subtilis* is inhibited by sulfhydryl, carbonyl, and amino group reagents as well as by anions indicative of the calcium-requiring nature of the enzyme (7).

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After inhibition experiments were carried out with appropriate reagents under the conditions reported previously (16 hours at 37.5°C. and pH 6.0), the extent of each of the inhibitions was determined by two analytical methods, one dependent upon liquefaction (8), and the other upon dextrinization (6).

The tabulated results show that the enzyme solutions were inhibited to the same degree with respect to liquefying and dextrinizing potency. From these data it may be concluded that *B. subtilis* α-amylase so depends for its activity upon 4 of its essential constituents that the independent existence of either liquefying or dextrinizing activity is highly improbable.

TABLE I

Effects of Inhibitors upon Liquefaction and Dextrinization

Reagent	Molarity	Activity remaining	
		Liquefying	Dextrinizing
		Per cent	Per cent
– –	––	100	100
Ag^+	1×10^{-3}	46	50
$NH_2CONHNH_2$	1×10^{-2}	14	13
HNO_2	1×10^{-1}	84	82
$(COOH)_2$	1×10^{-2}	29	28

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Studies on the Nutritional Requirements
of *Schizosaccharomyces pombe* (2478)

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INTRODUCTION

During an investigation on yeasts it was noted that *Schizosaccharomyces pombe* (2478) did not develop readily in some of the synthetic media used. Reports in the literature by Copping (1929), Stantial (1932), Nielson and Fang (1937), Donovick (1940) and Burkholder, McVeigh and Moyer (1944), have indicated some of the vitamin requirements of this yeast, but no systematic study has been published. The purpose of this study was to determine the nutritional requirements of this species of the *Schizosaccharomyces*.

EXPERIMENTAL

The culture chosen for this study was *Schizosaccharomyces pombe* (2478), which had been carried on dextrose-tryptone-yeast extract agar. Inocula for the experiments were grown on this agar 48–72 hours, washed off, centrifuged and resuspended in water. The inoculum for each set of flasks was prepared by diluting a suspension prepared as above described so that it had a light transmission of 80 to 83%. One ml. of this suspension, containing an average of 2,000,000 cells/ml., was used to inoculate each flask.

Tests were conducted in 500 ml. flasks using 100 ml. of media per flask. All results reported are on flasks which, after inoculation, were placed on a shaker having a 2½ inch stroke and running at 96 strokes per minute. Readings were usually taken after 48 to 72 hours; however, under certain conditions, where total growth was desired, they were allowed to continue to 96–120 hours. The temperature of incubation ranged between 25°–28°C. All media were prepared in carefully cleaned flasks and sterilized by heating ten minutes at 120°C. in the autoclave.

Responses were measured throughout this work by two methods; namely, hemocytometer counts and turbidity methods. For turbidimetric determinations a Lumetron photoelectric colorimeter was used and results reported as per cent light transmitted. Comparable results could be obtained by either technic.

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Four media were chosen for the original nutritional work. They consisted of Czapek-Dox medium with the addition of ammonium sulfate, Williams and Saunders biotin assay medium (1942, 1943), Rogosa's medium for lactose-fermenting yeasts (1943), and Schultz, Atkin and Frey's medium for pyridoxine determination (1936). Counts obtained from the four media were all low as compared with these same media when 0.3% yeast extract was added, even after prolonged incubation. These results indicated that all 4 media lacked something supplied by the addition of yeast extract in this concentration. Best results were obtained using the medium of Schultz, Atkin and Frey; consequently, it was chosen for further work. Table I gives the amount used per liter single strength medium.

TABLE I

Basal Medium for Growth of Schizosaccharomyces pombe 2478

Amount per liter single strength medium

Dextrose	50 g.	*Citrate buffer	5 ml.
KH_2PO_4	550 mg.	Acid-hydrolyzed casein,	
KCl	425 mg.	adjusted to pII 5.5	5.0 g.
$CaCl_2 \cdot 2H_2O$	125 mg.	Water to make 1000 ml.	
$MgSO_4 \cdot 7H_2O$	125 mg.	**Vitamins	
$FeCl_3 \cdot 6H_2O$	2.5 mg.		
$MnSO_4 \cdot 4H_2O$	2.5 mg.		
	Reaction adjusted to pH 5.5		

* Citrate buffer—100 g. potassium citrate, 20 g. citric acid, water added to make 1000 ml.

** To make this basal medium complete for maximum growth of *Schizosaccharomyces pombe* 2478, the following vitamins were found to be required/l.: thiamine, 500 γ; biotin, 1.0 γ; nicotinic acid, 1000 γ; calcium pantothenate, 1000 γ; and inositol, 10,000 γ.

The following vitamins were tested in this basal medium: inositol, nicotinic acid, biotin, *p*-aminobenzoic acid, thiamine, riboflavin, calcium pantothenate, pyridoxine and folic acid (Vitamin Bc). In these tests 50 ml. double strength basal medium was added to each flask and, after the addition of the vitamins, the volume was adjusted to 100 ml. by addition of distilled water. All results are reported on the basis of this volume of media. Both turbidity and hemocytometer counts were used to determine the results which are recorded in Table II.

With the basal medium used the above results indicate a requirement for inositol, nicotinic acid, biotin, and calcium pantothenate. Growth was not affected by the omission of *p*-aminobenzoic acid, riboflavin, pyridoxine or folic acid. It was concluded that the addition of these vitamins was not required by this organism under the conditions of this test. Thiamine held an intermediate position. Growth was obtained when thiamine was omitted; however, growth was increased when it was included in the medium.

TABLE II

Vitamin Requirements of Schizosaccharomyces pombe 2478

Treatment	72 hours incubation Turbidity Per cent transmission	Hemocytometer count/ml.
All vitamins	6.0	400,000,000
Minus inositol	97.0	900,000
Minus nicotinic acid	97.0	1,000,000
Minus biotin	97.0	1,100,000
Minus p-aminobenzoic acid	7.0	520,000,000
Minus thiamine	20.0	200,000,000
Minus riboflavin	7.0	500,000,000
Minus calcium pantothenate	97.0	850,000
Minus pyridoxine	7.0	500,000,000
Minus folic acid	7.0	420,000,000

To check the validity of the above results, flasks of basal medium were prepared and the 5 vitamins added. These flasks were compared with similar flasks containing all vitamins and vitamins supplemented with yeast extract. Tests run in 48 hours indicated a more rapid growth in medium supplemented with yeast extract than in either of the other two media. Growth in the flasks containing the 5 vitamins equaled that in the flasks containing all the vitamins. After 96 hours incubation, all flasks gave essentially the same number of yeast cells by hemocytometer counts.

The concentration of vitamins used to determine the requirement of this organism had been chosen from the amounts used in other work. To determine the actual amount of each vitamin required, basal medium was prepared and vitamins in varying amounts were added. Basal medium plus vitamins plus yeast extract was used as a control. Readings were made in 48 hours. From these tests the following amounts of vitamins were found to be satisfactory for maximum growth in this basal medium: thiamine, 25 γ, calcium pantothenate, 100 γ, nicotinic acid, 100 γ, bitoin, 0.1 γ, and inositol, 1000 γ/100 ml. of medium. In these tests it was observed that the basal medium with the addition of yeast extract alone does not supply sufficient vitamins for maximum growth.

Since the vitamin requirement of this yeast has been determined, it seemed of interest to study the activity of various fractions or modifications of the required vitamins. Consequently, the basal medium was prepared containing all the requirements of the yeast with the exception of the vitamin being tested. Results were compared with a medium containing all the known requirements. The following substances were tested: nicotinamide, methyl ester of biotin, pimelic acid, β-alanine, 4-methyl-5-β'-hydroxyethylthiazole, 2-methyl-5-ethoxymethyl-6-aminopyrimidine, 2-methyl-4-amino-5-hydroxymethyl-pyrimidine, 2-methyl-4-amino-5-aminomethylpyrimidine, and cocarboxylase.[1] These compounds were added to the medium in molar

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[1] We wish to thank Merck and Company for supplying the pyrimidines, the thiazole, and the cocarboxylase used in this work.

amounts equivalent to the amount of vitamin they were replacing. The following results represent readings after 48 and 72 hours. For this test one ml. of washed inoculum, containing 1,500,000 cells/ml., was used. Both hemocytometer counts and turbidity determinations are recorded in Table III.

TABLE III

Response of Schizosaccharomyces pombe 2478 to Various Fractions of Vitamins or Modification of the Vitamins

Treatment		Results			
		48 hours		72 hours	
Material omitted	Material substituted	Count	Turbidity Per cent trans.	Count	Turbidity Per cent trans.
None	None	125,000,000	15	315,000,000	8
Nicotinic acid	Nicotinamide	125,000,000	14	335,000,000	7
Biotin	Methyl ester biotin	30,000,000	50	75,000,000	43
Biotin	Pimelic acid	1,500,000	97	3,000,000	94
Calcium pantothenate	β-Alanine	135,000,000	16	280,000,000	8
Thiamine	None	28,000,000	46	215,000,000	11
Thiamine	*Thiazole	115,000,000	15	385,000,000	8
Thiamine	**Pyrimidine 1	27,000,000	43	285,000,000	9
Thiamine	Pyrimidine 2	20,000,000	53	315,000,000	7
Thiamine	Pyrimidine 3	20,000,000	54	300,000,000	7
Thiamine	Cocarboxylase	170,000,000	14	315,000,000	8
Thiamine	Thiazole and pyrimidine 1	—	15	265,000,000	8
Thiamine	Thizaole and pyrimidine 2	—	16	305,000,000	8
Thiamine	Thizaole and pyrimidine 3	—	15	330,000,000	8

* Thiazole, 4-methyl-5-β'-hydroxyethyl thiazole.
** Pyrimidine 1, 2-methyl-5-ethoxymethyl-6-aminopyrimidine.
  Pyrimidine 2, 2-methyl-4-amino-5-hydroxymethylpyrimidine.
  Pyrimidine 3, 2-methyl-4-amino-5-aminomethylpyrimidine.

After 5 days incubation the same relative results were obtained as were reported above for 48 and 72 hours. It appears that in this basal medium nicotinamide was used by this organism as readily as nicotinic acid. The methyl ester of biotin cannot be substituted for biotin. The

yeast seems to be unable to rapidly hydrolyze the ester to obtain the biotin it requires. Pimelic acid cannot be substituted for biotin. β-Alanine was as effective in promoting growth as was pantothenic acid. This yeast is able to use thiazole and cocarboxylase as readily as thiamine. When the pyrimidine fractions were supplied, growth was no better than when thiamine was completely omitted. The combination of thiazole and pyrimidines gave no better results than thiazole alone. It appears that the thiazole fraction of thiamine is synthesized slowly. When this is applied, the growth is as rapid as when thiamine itself is added.

Studies were conducted to determine the possibility of substituting ammonium sulfate for hydrolyzed casein, supplied in the original medium. Similar media were prepared, except that ammonium sulfate was used as the nitrogen source instead of hydrolyzed casein. The results in 48 hours gave a hemocytometer count of 453,000,000 /ml. for the hydrolyzed casein. Three concentrations of ammonium sulfate, namely, 0.1, 0.3, and 0.5%, gave essentially the same counts, approximately 220,000,000/ml, or about half that of hydrolyzed casein. Extending the incubation period to 96–120 hours resulted in equal growth in flasks with either nitrogen source. Thus, more rapid growth was obtained using hydrolyzed casein as the nitrogen source, but total growth was the same in all flasks. Combinations of hydrolyzed casein and ammonium sulfate did not·increase the rate of growth or total growth over that of hydrolyzed casein alone.

To test the completeness of the medium resulting from this work continuous transfers were set up. Four types of media were prepared for this experiment: Medium 1, regular medium as indicated above; medium 2, same as medium 1 except 0.1% ammonium sulfate substituted for hydrolyzed casein; medium 3, regular medium minus thiamine; medium 4, regular medium with addition of 0.3% yeast extract. The original inoculum was prepared as usual. After the first transfer the inocula for each medium were prepared from the growth obtained in that particular medium. After centrifuging and resuspending in water, 2,000,000 cells were inoculated into respective media from which they were obtained. Growth was maintained in all four media throughout six transfers. Readings were made at the end of each 48 hours. The average number of cells per ml. of these four media for the six transfers follows: Medium 1, 228 million; medium 2, 100 million; medium 3, 43 million; medium 4, 349 million. The highest average count was maintained in the regular medium plus 0.3% yeast extract (medium 4). Regular medium (medium 1) gave slightly lower counts. The medium in which ammonium sulfate was substituted for hydrolyzed casein and the medium with no thiamine (medium 3) gave definitely lower counts for each transfer than the other two, despite the fact that all flasks receive the same amount of inoculation. Attempts to obtain increased rate of growth by doubling the amount of hydrolyzed casein, increasing the amount of vitamins, or adding pyridoxine, folic acid, choline, adenine, guanine, uracil, asparagin, or amino acid mixtures, did not succeed in producing growth in the regular medium equal to that obtained when 0.3% yeast extract was added.

DISCUSSION

This study indicates that, under the conditions of our experiments, *Schizosaccharomyces pombe* (2478) requires biotin, nicotinic acid, calcium pantothenate and inositol. These results are in accord with those reported by Burkholder *et al.* for another strain of this yeast. Although growth was maintained without thiamine in the medium used in this work, the rate of growth was increased by its addition. For maximum results the following amounts of vitamins were found essential: inositol 1000 γ, nicotinic acid 100 γ, calcium pantothenate 100 γ, biotin 0.1 γ, and thiamin 25 γ/100 ml. of medium. Riboflavin, pyridoxine, *p*-aminobenzoic acid, or folic acid, had no effect on growth of this yeast. It was found that nicotinamide could substitute for nicotinic acid; β-alanine for calcium pantothenate; the thiazole (4-methyl-5β'-hydroxyethyl thiazole) and cocarboxylase for thiamine. The methyl ester of biotin was not as readily used as biotin, and pimelic acid had no biotin activity.

The substitution of ammonium sulfate for hydrolyzed casein as a nitrogen source resulted in a slower rate of growth of this yeast. If the incubation time was prolonged to 96–120 hours, the total growth was the same in both media. The addition of yeast extract in a concentration of 0.3% to regular medium gave a more rapid rate of growth than where it is omitted. However, yeast extract added to basal medium in which the required vitamins were omitted does not give as good growth as where these vitamins are also supplied. None of the modifications of the regular hydrolyzed casein medium tried in this work produced a rate of growth equal to that obtained by this relatively large amount of yeast extract.

SUMMARY

With the basal medium used in this work, it was found that *Schizosaccharomyces pombe* (2478) required biotin, calcium pantothenate, nicotinic acid and inositol for growth. The addition of thiamine increased the rate of growth. On the basis of 100 ml. of Schultz, Atkin and Frey's medium, this yeast requires 0.1 γ of biotin, 100 γ of nicotinic acid, 100 γ of calcium pantothenate, 1000 γ of inositol, and 25 γ of thiamine for maximum growth.

Nicotinamide, β-alanine and thiazole can be substituted for their respective vitamins without affecting growth of this organism.

Hydrolyzed casein serves as a better source of nitrogen than ammonium sulfate when rate of growth studies are made. Both are of equal value if total growth is measured after an extended incubation period.

Yeast extract, when added to the medium in 0.3% strength, appears to contain some stimulative substance or substances not present in hydrolyzed casein or in the vitamin mixtures used.

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The Effects of Some Metallic Ions on Lactobacilli

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INTRODUCTION

Intensive studies on the nutritional requirements of the organisms
used in microbiological assays have led to a steady improvement in
assay media. Relatively little attention, however, has been given to the
adequacy of the inorganic constituents provided.

In 1945 Snell (1) reported that there had been no detailed investiga-
tion of the mineral nutrition of the lactic acid bacteria. Möller (2)
showed manganese to be essential to the Orla-Jensen strain of *Lacto-
bacillus plantarum*. Woolley (3) demonstrated the increased growth rate
of *Lactobacillus casei* upon the addition of manganese to a peptone-
glucose medium.

While this paper was being written, Bentley *et al.* (4) published a
microbiological method for the determination of manganese based on
the requirements of *L. arabinosus* for that ion, and MacLeod and Snell
(5) demonstrated the essential nature of Mn, Mg and K for several
lactic acid bacteria.

This study is concerned with an analysis of the inorganic composition
of an assay medium and the effects of added ions on the growth and
acid production of three assay organisms.

MATERIALS AND METHODS

The cultures used in this work were *Lactobacillus casei* 7469, *Lactobacillus arabino-
sus* 17-5, and *Lactobacillus delbrueckii* LD-5. Stocks were obtained from the American

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Type Culture Collection and transferred weekly on a Difco glucose-yeast extract agar to which were added 5 g. of liver extract/l.

The Landy and Dicken (6) medium for vitamin assay was chosen for the experimental work. Inocula, grown in this medium for 24 hours, were standardized by dilution to a transmittance of 50 at the 590 mμ setting on the Coleman spectrophotometer and were routinely employed in a concentration of 1% of the volume of medium to be inoculated. All cultures were incubated at 37°C. Turbidity was measured at 590 mμ with a Coleman spectrophotometer. Titratable acidity was determined on 10 ml. amounts of culture and expressed in terms of ml. $N/10$ NaOH necessary for neutralization.

Fermentations were carried out on 200 ml. quantities of media in Erlenmeyer flasks. Lactic acid was determined by the method of Friedemann and Graeser (7); residual glucose by the method of Somogyi (8). Volatile acid, alcohol, and acetone determinations were made, but the data are not reported because there was no significant difference between the experimental and control cultures. Fermentation balances were calculated for each flask.

Since the problem of contamination in trace metal analyses is a serious one, the warnings and recommendations of Sandell (9) have been heeded in these investigations. Only reagent grade chemicals were used. Diluting fluids were redistilled acids and ammonium hydroxide, as well as dithizone-treated organic solvents. Distilled water was redistilled through an all-glass apparatus. All glassware employed in analyses was Pyrex. The cleaning regime consisted of overnight contact with aqua regia, followed by alcoholic potassium hydroxide. The vessels were rinsed 5 times with distilled water and autoclaved. After cooling, they were rinsed 3 times with fresh glass-distilled water. Washings were tested with dithizone. A new porcelain evaporating dish was used for each ashing procedure. Reagent blanks were run routinely.

Two-liter quantities of Landy and Dicken medium were ashed at 500°C. Two ml. of concentrated sulfuric acid were added during the concentration of these solutions to prevent volatilization of metallic elements during the ashing. The ash was taken up in a mixture of dilute HCl and HClO$_4$. The resulting solution was analyzed for copper, cobalt, manganese, molybdenum, zinc, and magnesium according to the methods outlined by Parks et al. (10). Iron was determined by the dipyridyl method as modified by Kitzes et al. (11). The method recommended by Sandell (9) was used in estimating cadmium.

EXPERIMENTAL FINDINGS

a. Analysis of Landy and Dicken Medium for Metallic Ions

An effort was made to determine the quantities of various metals present in Landy and Dicken medium as it would be made up in an assay laboratory. While it is obviously impossible to duplicate conditions of contamination, or small errors in calibration of equipment, it was thought that some approximation of the amounts of metals likely to be present could be achieved. Table I shows the range of content, in mg./l., of 8 cations in 4 separate batches of medium.

TABLE I

Analysis of Landy and Dicken Medium for Metallic Ions

	Fe	Mn	Zn	Mg	Cd	Mo	Co	Cu
Mg./l added in Salts B of formula:	1.0	0.9		9.87				
Mg./l determined by chemical analysis:	1.35	2.25	1.03	10.52	0.022	0[a]	0[a]	0[a]
	2.05	1.415	3.58	10.44	0.037	0	0	0
	1.12	2.67	3.14	16.05	0.027	0	0	0
	1.80	1.78	2.48	11.60	0.034	0	0	0.0013

[a] Methods employed in these determinations are not sensitive to less than 1 γ.

It is apparent from these data that considerable amounts of iron, manganese, and magnesium are picked up in excess of those added in Salt Solution B of the formula. Molybdenum and cobalt are notably absent, or at least not detectable by the chosen method of assay. Copper tests were negative in four media out of five.

b. Copper

Copper sulfate was added to a batch of medium giving no test for that metal. The effect of the sulfate ion was controlled with sodium sulfate. Table II shows the effect of copper on the 3 lactobacilli. Addition of 0.01–0.1 mg. of Cu/l., a range expected to give stimulation if the organisms have an unsatisfied copper requirement, provided no significant increase in titratable acidity over the control medium. Figures shown in Table II are averages of results obtained on triplicate samples of each copper level and each incubation period. The concomitant turbidimetric and fermentation studies indicate that both growth and acid production were affected by the higher levels of copper. The organisms incubated 72 hours are, in some cases, able to overcome the inhibitory effect of the copper ion.

c. Cobalt

Hydrated cobalt chloride was added aseptically in amounts from 0.01–100 mg. to a medium which gave no test for the ion. Chloride effects were controlled with the sodium salt. Addition of small amounts

TABLE II

The Effect of Copper On Three Lactobacilli

	L. casei						L. delbrueckii						L. arabinosus					
	Turbidity		N/10 NaOH		Lactic acid		Turbidity		N/10 NaOH		Lactic acid		Turbidity		N/10 NaOH		Lactic acid	
Hours incub.	24	72	24	72	24	72	24	72	24	72	24	72	24	72	24	72	24	72
Cu mg./l.																		
0	13	6	9.4	9.9	501	543	17	12	9.6	10	513	576	6	6	9.9	10	583	584.7
1.0	23	6	4.5	9.8	343	543	17	13	9.5	10	513	576	7	6	9.9	10	583	584.7
5.0	52	9	2.9	9.4	119	531	32	22	7.3	8.1	396	445	9	6	9.9	10	583	584.7
15.0	55	20	2.9	6.8	119	360	42	42	4.0	5.3	229	264						
20.0	88	39	1.5	3.0	46	119	61	89	2.1	2.8	126	153	14	7	9.7	9.9	579	584
25.0	100	100	0.3	0.3			100	100	0.3	0.3								
50.0													19	9	9.6	9.9	568	583
65.0													28	15	7.0	9.6	420	570
80.0													42	22	5.0	8.5	315	528.9
Uninoculated							100		0.3		0							

of the cobalt ion gave rise to no stimulation; increasing concentrations produced a decline in growth and acid production. As in the experiments with copper, turbidity, titratable acidity, and lactic acid reflected the same trend. For this reason, only turbidity data are given in Table III.

d. Molybdenum

Neutralized molybdenum pentachloride, added to a medium giving no test for Mo, produced no effect on acid production or growth of the 3 lactobacilli until the concentration exceeded 170 mg./l.

e. Zinc

Table I indicates that the zinc content of the media examined varied from 1 to 3.5 mg. of Zn/l. Accordingly, media with high and low zinc contents were selected for comparison. The effect of zinc sulfate added to the medium already containing 1 mg./l. is seen in Table IV. Slight stimulation resulted from the addition of 1 mg. or less zinc. This

TABLE III

The Effect of Cobalt on Lactobacilli

	L. casei		L. delbrueckii		L. arabinosus	
Hours incubation	24	72	24	72	24	72
Co.	Turbidity					
mg./l.						
0	12	12	22	13	7	7
20	15	12	34	15	7	7
40	42	14	52	13	15	7
50	85	23	77	51	66	7
60	91	48				
80	100	100	79	72	87	7
00			100	100	90	7
Uninoculated	100					

effect was not seen when zinc was added to the medium already containing 3.5 mg. The addition of 20 mg. or more produced a precipitate which probably contributed to rendering the medium unfavorable for the organisms.

TABLE IV

The Effect of Zinc on Lactobacilli as Shown by Titratable Acidity

	L. casei		L. delbrueckii		L. arabinosus	
Zn	Hours of incubation					
	24	72	24	72	24	72
mg./l.						
0[a]	8.4	9.8	4.5	9.3	9.8	9.8
1.0	9.0	9.8	5.0	9.8	9.8	9.8
5.0	8.2	9.7	5.0	9.8	9.8	9.8
15.0	5.2	9.7	3.5	9.8	9.0	9.8
25.0	2.8	9.7	1.9	9.2	8.4	9.2
50.0	0.4[b]	0.4	0.4	4.1	1.8	8.1
75.0					0.4	0.4

[a] The medium was shown by analysis to contain 1.03 mg. of zinc/l.
[b] A titration of 0.4 is equivalent to that of the uninoculated medium.

f. Cadmium

Table V shows the effect of addition of hydrated cadmium chloride to a medium containing 34.63 γ of Cd/l. After 24 hours incubation,

TABLE V

The Effect of Cadmium on Lactobacilli as Shown by Titratable Acidities

Cd[a]	L. casei		L. delbrueckii		L. arabinosus	
	Hours of incubation					
	48	72	48	72	48	72
mg./l.						
0[a]	10.2	10.4	9.9	10.4	10.4	10.4
0 2	7.4	10.4	9.9	10.4	9.2	10.4
0.4	3.0	6.8	9.7	10.4	6.7	10.2
0.6	1.3	2.5	9.2	10.4	4.2	10.0
0.8	0.6	1.3	7.7	9.8	0.3	8.4
1.0	0.3[b]	0.3	5.0	9.6	0.3	0.3
5.0	0.3	0.3	1.1	4.0		
10.0			0.3	0.3		

[a] The medium contained 34.63 γ of Cd/l.
[b] 0.3 ml. represents the titration of uninoculated medium.

only the control tubes showed turbidity; therefore, measurements were not made until 48 hours. This ion was more markedly inhibitory than the others tested.

g. Omission of Ions

Salt Solution B of the Landy and Dicken medium contains, in addition to sodium chloride, the sulfates of manganese, iron, and magnesium. When this solution is added to the medium in the required amounts, 1 mg. Fe, 0.98 mg. Mn, and 9.87 mg. Mg are supplied/l. Addition of larger amounts of these ions to the medium produced no stimulation.

In these experiments, the iron, manganese, and magnesium were omitted one by one, in pairs, and altogether. The effects of these alterations of 24-hour titratable acidities can be seen in Table VI. Four separate batches of medium are represented. Each figure in the table was obtained from triplicate samples on each batch of medium. The

TABLE VI

Effect of Variation in Salts B Added to Landy and Dicken Medium

Alteration of B salts	L. casei	L. delbrueckii	L. arabinosus
	Ml. $N/10$ NaOH above or below amount necessary for neutralization of complete medium		
− Mg and Mn	+0.8	+0.9	−0.35
− Fe and Mn	+0.66	+0.95	−0.8
− Fe and Mg	+0.6	+0.75	−0.25
− Fe	+0.2	+0.7	−0.4
− Mg	−0.2	−0.5	−0.1
− Mn	+0.17	+0.7	−0.6
− B Salts	+0.1	+0.2	−0.7

These figures are derived from triplicate samples on 4 separate batches of medium.

basic medium, prior to the addition of any of the ions contained in the B salts, averaged 488 γ of iron, 493.3 γ of manganese, and 7.45 mg of magnesium/l.

From Table VI it is apparent that the omission of ions from the medium was, in general, detrimental to *L. arabinosus*. The contaminating Mn and Mg were apparently sufficient to satisfy the requirements of *L. casei* and *L. delbrueckii* LD-5 (5). The acid produced by these two organisms was more under these conditions than when Mn and Mg were supplied according to the formula.

DISCUSSION

The question of whether or not the inorganic content of the medium may have an effect on the accuracy of vitamin assays prompted this investigation. In addition to the metals entering the medium from reagents and vessels, there is the possibility that certain ions may be incorporated into the medium in significant quantities along with the material being assayed. It is well known, for example, that the inorganic content of vegetation reflects that of the soil on which it is grown. Thus, it was thought possible that occasional high or low results in assays of certain foodstuffs might be attributable to ion content of the sample. In the light of the above results, in connection with the particular ions studied, this seems unlikely except in certain isolated instances. A survey of literature was undertaken to determine the mineral

content of 50 plant and animal tissues. The amounts of such tissues likely to be incorporated in assay tubes in the estimation of riboflavin were calculated, and from these figures were obtained the concentrations of zinc, copper, and cobalt which would thus be added to the medium. Only in the case of tomatoes containing 50 p.p.m. copper, and liver containing 245 p.p.m. zinc, both cited by Calvery (12), would the ion content of the sample have a possible effect on the results of that assay. Concentrations of manganese and iron would have to exceed 50 and 100 mg./l., respectively, before acid production would be affected adversely. These figures apply only to the Landy and Dicken medium.

Omission of certain ions of the B salts, as shown in Table VI, has produced some interesting results. Only the omission of magnesium and of all the B salts caused *L. casei* and *L. delbrueckii* to produce less acid. The increased acidity produced by the omission of magnesium and manganese, both of which seem to be necessary to the *Lactobacilli*, suggests the possibility that the Landy and Dicken formula does not provide an optimum ion balance for these organisms.

SUMMARY

1. The inorganic content of Landy and Dicken vitamin assay medium, as it might be made up in an assay laboratory, was determined. In addition to the Fe, Mn, and Mg supplied by formula, Zn and Cd were found.

2. Ions not detectable in the ash of the medium (Cu, Co, Mo) produced no stimulation of reproduction or lactic acid formation when added to the medium. Increased concentrations of Cu and Co inhibited cellular activity.

3. Reduction in lactic acid production caused by large quantities of cobalt and copper appeared to be a function of decreased reproduction of the cultures.

4. Prolonged incubations (72 hours) allowed the organisms to overcome the initial lag in many cases and obscured the effect shown in 24 hours.

5. Alterations of the Landy and Dicken medium with regard to inorganic content permitted higher acid production by *L. casei* and *L. delbrueckii* than the original formula. The modifications were not favorable to *L. arabinosus*.

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The Colorimetric Determination of Isoleucine in Biological Products [1]

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INTRODUCTION

The estimation of valine and leucine in protein hydrolyzates by the scheme proposed by Fromageot and co-workers (1, 2) is based on the colorimetric measurement of the acetone formed by the degradative oxidation of these amino acids by the following reactions:

Valine

$$
\begin{array}{c}
CH_3 \\
\diagdown \\
CH \cdot CH \cdot COOH \\
\diagup \\
CH_3
\end{array}
\begin{array}{c}
NH_2 \\
\cdot \\
\end{array}
\ \xrightarrow{\ HONO\ }\
\begin{array}{c}
CH_3 \\
\diagdown \\
CH \cdot CH \cdot COOH \\
\diagup \\
CH_3
\end{array}
\begin{array}{c}
OH \\
\cdot \\
\end{array}
\ \xrightarrow{\ CrO_3\ }\
\begin{array}{c}
CH_3 \\
\diagdown \\
CO + \text{other products.} \\
\diagup \\
CH_3
\end{array}
$$

Leucine

$$
\begin{array}{c}
CH_3 \\
\diagdown \\
CH \cdot CH_2 \cdot CH \cdot COOH \\
\diagup \\
CH_3
\end{array}
\begin{array}{c}
NH_2 \\
\cdot \\
\end{array}
\ \xrightarrow{\ HONO\ }\
\begin{array}{c}
CH_3 \\
\diagdown \\
CH \cdot CH_2 \cdot CH \cdot COOH \\
\diagup \\
CH_3
\end{array}
\begin{array}{c}
OH \\
\cdot \\
\end{array}
\ \xrightarrow{\ CrO_3\ }\
\begin{array}{c}
CH_3 \\
\diagdown \\
CO + \text{other products.} \\
\diagup \\
CH_3
\end{array}
$$
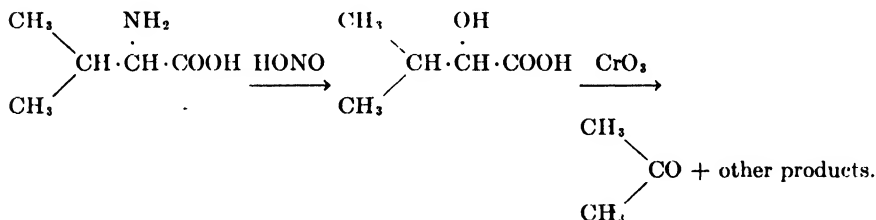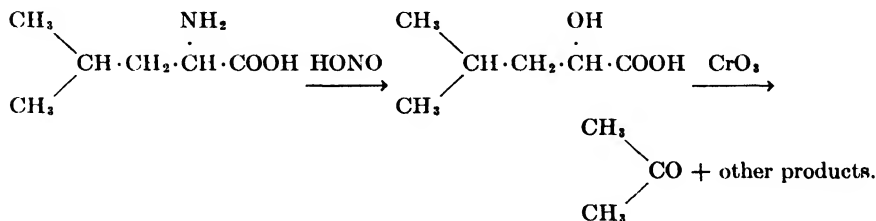
Block (3) has extended this principle to the estimation of isoleucine as ethylmethyl ketone derived by analogous reactions:

ı   21

Isoleucine

$$CH_3 \diagdown \quad NH_2 \atop C_2H_5 \diagup CH \cdot CH \cdot COOH \quad HONO \longrightarrow \quad CH_3 \diagdown \quad OH \atop C_2H_5 \diagup CH \cdot CH \cdot COOH \quad CrO_3 \longrightarrow$$

$$CH_3 \diagdown \atop C_2H_5 \diagup CO + \text{other products.}$$
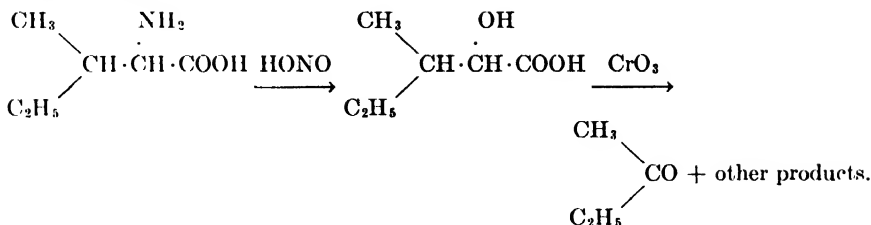
The analytical success of these schemes is obviously dependent on three factors: (a) use of oxidation reactions which yield constant amounts of the ketones from the corresponding amino acids, (b) adequate differential oxidation mechanisms to permit establishment of mathematical equations for the calculation of the amounts of leucine and valine from the yield of acetone, and (c) suitable procedures for the accurate estimation of the acetone and ethylmethyl ketone formed by these reactions.

In his work Fromageot has utilized the chromic acid-acetic acid system for the oxidation of the corresponding hydroxy acids of valine and leucine to acetone. A suitable difference in yield of acetone from the two amino acids for purposes of calculation was found by performing the oxidations at normal and at high pressures. Block (4) in his modification of the Fromageot scheme has instituted different systems of oxidation which are claimed to yield more reliable data. Our investigation of these methods, which was begun in 1942, has shown that the yield of acetone from valine and leucine by the Fromageot or Block procedures is not sufficiently constant to render the data suitable for analytical interpretation. This experience led us to attempt the permanganate oxidations at acid levels high enough to be unaffected by hydrogen ion fluctuations produced by the reaction. Although we were successful in this approach, and found that suitable differences in the yield of acetone from valine and leucine could be attained by performing the permanganate oxidation at 0.3 N and 0.5 N with respect to H_2SO_4, the yield of acetone from valine and leucine, in particular, was too low to permit the establishment of the necessary mathematical equations. Concurrent experiments, however, showed that the yield of ethylmethyl ketone derived from the chromic acid oxidation of the hydroxy analog of isoleucine, under conditions of high but constant acidity and controlled heating, is sufficiently reproducible for analytical purposes. It was also found that the quantitative characteristics of the reaction could be more readily realized by performing the oxidations and simultaneous distillations in a modified form of the apparatus devised by Friedemann (5) for the determination of lactic acid. Difficulties arising from the use of the available techniques for measuring ethylmethyl ketone (3, 6) were resolved ultimately by the establishment of a new method for the direct determination of ethylmethyl ketone based on a quantitative adaptation of the acid vanillin color reaction originally described for this ketone by Rosenthaler (7), which does not appear to be interfered with

by other products resulting from the chromic acid oxidation of deaminized protein hydrolyzates.

Analyses of 10 different standard solutions of DL-isoleucine in which the standardized oxidation conditions, improved reaction apparatus, and direct colorimetric procedure for the ketone were employed, yielded 17.33 ± 0.24% of the theoretical ethylmethyl ketone equivalent of isoleucine. This oxidation value is approximately one-third of that reported by Block but is so reproducible as to introduce an error of only ±8% in the final result. The practical analytical value of the proposed method was subsequently established to our satisfaction by recovery tests and by its application to mixtures of crystalline amino acids. Analyses of the hydrolyzates of a number of biological products by the proposed method were performed and the results are compared with the available data. In this connection we were interested to note from our own data that enzymatic and HCl digests of proteins contained greater amounts of isoleucine than did the sulfuric acid hydrolyzates of the same proteins, indicating that the removal of this acid as the calcium salt entails an inevitable loss of this essential amino acid.

Attention is called to some advantages of the chemical procedure to be described here which are not inherent in the currently popular microbiological techniques: (a) the determination of isoleucine can be performed in laboratories not equipped with microbiological facilities, (b) the analytical results can be obtained within 2 hours, in contrast to the 24 hours or more required for the microbiological measurement, and (c) the chemical technique measures both D- and L-isoleucine rather than L-isoleucine alone found by the microbiological techniques.

EXPERIMENTAL

Reagents

Sulfuric Acid. 0.5 N and 2 N solutions and the concentrated reagent.

Sodium Nitrite. A 50% solution is required which should be prepared fresh at least once a week.

Urea. 15% solution.

Sodium Bisulfite. A freshly prepared 5% solution is needed.

Oxidizing Reagent. This consists of a 1:1 mixture of 4 N sulfuric acid and 4 N potassium dichromate. This reagent is stable and can be prepared in liter quantities. It should be stored, of course, in ground glass-stoppered pyrex bottles.

Acid-vanillin Reagent. 250 mg. of vanillin (E. K. Co. No. 273) are dissolved in 2.5 cc. of absolute methanol and this solution diluted to a 25 cc. volume with concentrated HCl. This reagent deteriorates rapidly on standing and should be prepared just prior to its use.

Isoleucine Standard. This is required to check the reagents and analytical technique. DL-Isoleucine, Merck, found to contain 10.66% N by micro Kjeldahl (theory, 10.68% N), was used, and solutions containing 10–15 mg. of the amino acid/cc. were found to be satisfactory for this purpose.

Apparatus

The structural details of this are shown diagrammatically in Fig. 1. It will be noted that the necessary parts can be either easily made in the laboratory or obtained readily
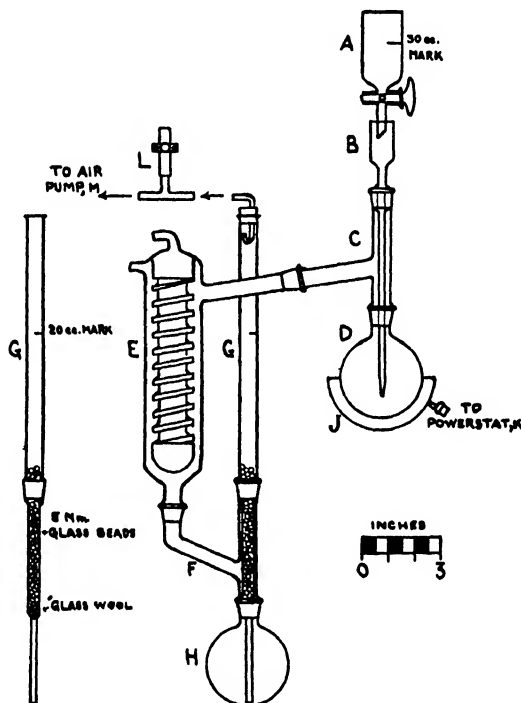
FIG. 1. Drawing of apparatus. All numbers refer to Corning catalog LP-24 and 24/40 standard taper interchangeable ground joints were used throughout. A, 60 cc. separatory funnel, 6420; B, delivery tube; C, connecting tube, 9000; D and H, 250 cc. boiling flasks, 4320; E, Friedrichs condenser, 2640; F, connecting tube, 9040; G, adsorption column packed with glass wool and 5 mm. glass beads as shown; J, Glas-Col heating mantle for 250 cc. flasks; K, Powerstat for temperature control; L, air pump, Fisher catalog No 90, 1–093; M, escape valve constructed from 6 mm. glass T tube, rubber hose and Fisher Castalloy hosecock.

as stock items of the Corning Glass Co. Uniform and controlled heating of the reaction flask (D) was achieved by the use of a Glas-Col heating mantle regulated by means of

a Powerstat. Constant negative pressure was attained by means of an electrically driven vacuum pump.

ANALYTICAL PROCEDURE

Hydrolysis of Proteins

Hydrolyzates were prepared by refluxing 50 g. of protein with 250 cc. of constant boiling HCl for 24 hours. The total N content of the hydrolyzate was determined directly by micro Kjedahl analysis (8); the excess of acid was then removed by concentration *in vacuo* and the humin separated by filtration. A nitrogen content of 10–30 mg./cc. of final product has been found a convenient concentration. Enzymatic digests have also been employed and appear to have isoleucine values comparable to HCl digests. The use of sulfuric acid digests which entail the removal of the acid as the barium or calcium salt is not recommended due to the loss of isoleucine which this process incurs.

Deamination and Oxidation of Protein Hydrolyzates

A sample of the digest not exceeding 10 cc. and containing 50–150 mg. of nitrogen is transferred into a 125 cc. Phillips beaker. If necessary, the volume of the sample is adjusted to 10 cc. and 5 cc. of 2 N H_2SO_4 and then 2 cc. of 50% sodium nitrite are added slowly with continuous agitation under a fume hood. This mixture is shaken intermittently and allowed to react at room temperature for 10 or 15 minutes, or until excessive bubbling has ceased. At this point, 3 cc. of 15% urea are added and the mixture heated gently to remove the residual nitrogen oxides.

This preparation, which contains the hydroxy analogs of the amino acids, is transferred quantitatively into the closed dropping funnel, A. To prevent any loss of sample, the reaction flask is carefully rinsed with 10 cc. of 0.5 N H_2SO_4, this washing is added to the contents of A and the volume of the mixture in A is adjusted to 30 cc. if necessary. Flasks D and H are removed from the apparatus and returned to the positions shown in Fig. 1 after 40 cc. of oxidizing reagent and 10 cc. of 5% sodium bisulfite had been added, respectively. After the oxidizing mixture in D was brought to a boil by setting the Powerstat, K, at 70, the suction pump, L, was turned on and the negative pressure regulated by escape valve, M, so that the sodium bisulfite solution in flask H was kept about 1 cm. above the glass beads in adsorption tower, G. The reaction mixture in A was then allowed to run into the boiling oxidizing reagent at such a rate that 5 minutes were required to complete the addition. Boiling is continued until 20 cc. of solution have distilled over, which is indicated by the rise of liquid to the mark on tube, G. The heating mantle, and suction pump were then switched off, the adsorption tower washed down with about 10 cc. of distilled water, and the distillate and washings transferred to a 50 cc. glass-stoppered graduated cylinder. The volume of this solution was brought to the 50 cc. mark and 1 cc. aliquots removed for the colorimetric determination of ethylmethyl ketone.

Colorimetric Estimation of Ethylmethyl Ketone[2]

One cc. aliquots of the distillate, containing 0.02–0.12 mg. of ketone are measured into 10 cc. graduated colorimeter tubes and treated with 1 cc. of acid vanillin solution and 1 cc. of concentrated sulfuric acid; both reagents are delivered from burettes. After thorough mixing, and standing for exactly 30 minutes, the reaction mixture is made to the 5 cc. mark with water and read immediately in the Klett-Summerson colorimeter with a No. 60 filter. A blank consisting of 1 cc. of distilled water plus the reagents is run in the same manner. In order to allow a reaction time of exactly 30 minutes for each determination when a series of assays is being carried out, the addition of reagents to each sample is spaced at 1 minute intervals and the readings then made in the same time intervals and order. The amount of EMK in the sample is best ascertained from a previously prepared calibration curve and the isoleucine equivalent calculated as shown below.

Calculations

On the basis of molecular weight, 1 mg. of isoleucine is equivalent to 0.55 mg. of EMK. However, since the oxidation of 1 mg. of isoleucine yields only 0.095 mg. of EMK, or 17.33% of theory, 1 mg. of the ketone is equivalent to 10.5 mg. of isoleucine. Thus, mg. of isoleucine in sample = mg. of EMK/cc. of distillate × 50 × 10.5.

RESULTS

The suitability of the modified Rosenthaler reaction for the estimation EMK was determined by submitting varying amounts of the ketone to the colorimetric test. The solutions for this purpose were prepared as follows: Approximately 1.25 cc. of freshly redistilled EMK were delivered into a weighed 15 cc. glass-stoppered weighing bottle containing approximately 7 g. of water. After the addition of EMK, the bottle was reweighed and the amount of ketone in solution determined from the weight difference. This solution was transferred quantitatively with several rinsings of the weighing bottle to a 1-liter volumetric flask and then made to the mark. The requisite dilutions were made from this stock solution. The results of these experiments are shown in Fig. 2. The linear relationship of color intensity to the amount of EMK is interpreted as evidence of the analytical adequacy of colorimetric procedure and of the validity of Beer's Law for the color reaction. It is also to be noted from Fig. 2 that the error of the colorimetric measurement averages about 6% over the range investigated. Since the colorimetric readings of the unknown samples are always compared with those of the EMK standard, a correction for this error

[2] Ethylmethyl ketone will hereafter be designated as EMK.

is not made in the calculations. However, a correction for the combined colorimetric and oxidation errors which amount to approximately ±8% must be applied to the final result.

The results of 10 runs indicated that, under the described standardized conditions of oxidations, wherein the concentration of H_2SO_4 varies only from 1.35 to 1.50 N, 1 mg. of DL-isoleucine yields 95.4 ± 1.8 γ of EMK. To test the specificity of both reactions a mixture containing 25 mg. each of the pure amino acids, L(+)-arginine HCl, L(+)-
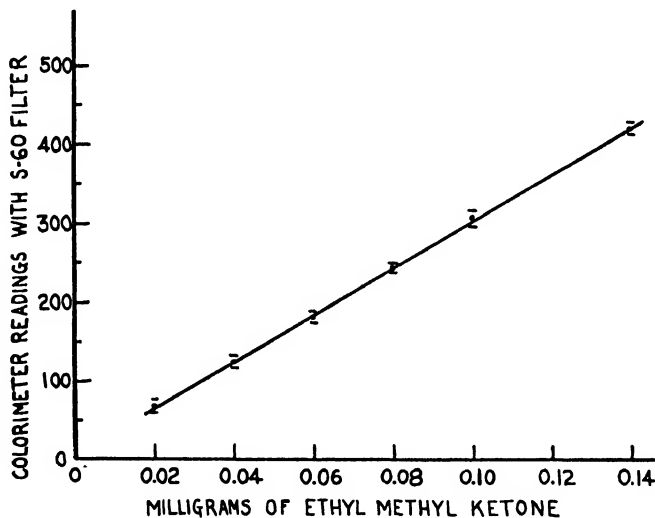
FIG. 2. Relation of color intensity of the modified Rosenthaler reaction to the amount of ethylmethyl ketone. Each point represents the average value and average deviation of 10 or more determinations.

histidine HCl, DL-methionine, L(−)-cystine, L(−)-tyrosine, DL − tryptophan, DL-phenylalanine, DL-serine, DL-threonine, L-leucine, DL-valine, DL-lysine HCl, DL-α-alanine and glycine, was submitted to the analytical procedure and found to yield negligible amounts of interfering substances. Acetone, acetaldehyde, and formaldehyde were also found not to interfere with the color reaction.

The applicability of the proposed method to the estimation of isoleucine in protein hydrolyzates was indicated by the favorable results obtained in recovery tests (Table I). The results of our analyses of the hydrolyzates of a number of biological substances are given in

TABLE I

Recovery of Isoleucine added to Protein Hydrolyzates

Product	Total N	Isoleucine added	Isoleucine content	Recovery of added isoleucine
	mg.	*mg.*	*mg.*	*per cent*
Amigen	34.0		14.7	
	34.0	36.6	52.5	102
Edamin	34.5		13.2	
	34.5	36.6	50.4	101
Casein (HCl digest)	51.2		22.6	
	51.2	36.6	58.8	99.8

TABLE II

Isoleucine Content of Hydrolyzates of Some Biological Substances

Biological product	Hydrolyzing agent	Uncorrected N content	Isoleucine N of total N
		per cent	*per cent*
Casein, Sheffield	HCl	12.50	4.70
Casein, Sheffield	H_2SO_4	12.50	3.39
Beef hemoglobin, Armour	HCl	14.00	0.76
Beef hemoglobin, Armour	H_2SO_4	14.00	0.58
Human hemoglobin	HCl	13.96	0.98
Human hemoglobin	H_2SO_4	13.96	0.76
Cattle fibrin, Wilson	HCl	15.10	5.82
Lactalbumin, Sheffield	HCl	11.70	5.82
Gelatin, U.S.P.	HCl	14.30	1.04
Amigen, Mead Johnson	Enzymes	11.80	4.62
Edamin, Sheffield	Enzymes	11.40	4.06
Brewers' yeast	HCl	4.92	2.05

Table II. It is to be noted that the sulfuric acid digests of casein, beef hemoglobin and human hemoglobin contain less isoleucine than the corresponding HCl digests. A sulfuric acid digest of casein without prior treatment with calcium hydroxide was found to contain 0.742% isoleucine N of total N; a figure which compares favorably with HCl digest value and suggests that the isoleucine loss incurred in the preparations arises from adsorption of the amino acid on $CaSO_4$ rather than selective destruction of the amino acid by H_2SO_4. Attention is called to the similarity in isoleucine content of Amigen, a pancreatic digest of casein, and the HCl digest of casein and dissimilarity of the isoleucine

content of Edamin, an enzymatic digest of lactalbumin and the HCl digest of lactalbumin. These observations emphasize the point that digestion and processing procedures may so modify the amino acid contour of protein as to alter their initial biological value (9).

A comparison of some of our results with those available in the literature discloses that our isoleucine values are higher than previously reported figures obtained by chemical techniques and microbiological methods (Table III).

TABLE III

Comparison of Results of Isoleucine Analyses of Proteins by Various Methods

Investigator	Methods	Isoleucine N of total N			
		Casein	Lact-albumin	Beef fibrin	Gelatin
		per cent	*per cent*	*per cent*	*per cent*
Authors	Chemical	4.7	5.8	5.8	1.1
Block (3)[a]	Chemical	3.4	2.8	3.3	0.7
Williamson (10)	Chemical	3.6	3.0		
Hier et al. (11)[a]	Microbiological	4.1	4.5	3.8	0.9

[a] The amino acid content of the proteins reported by these investigators have been reëvaluated to the nitrogen basis to facilitate comparison.

COMMENTS

It is to be noted that the yield of EMK from isoleucine by the described procedure is one-third of that reported by Block (3) and therefore necessitates the use of a greater amount of protein. However, when this greater need of material can be sustained our procedure is recommended for the greater accuracy and speed of operation which it affords. The higher isoleucine values obtained by our procedure (Table III) may be due in part to (a) the inadequacy of the oxidation technique and colorimetric reaction for EMK employed in the chemical method (3), (b) to the failure of the microbiological method to determine any D-isoleucine which may have been formed during hydrolysis of the protein (12), and (c) differences in the quality of protein.

SUMMARY

A colorimetric method for the determination of isoleucine based on chromic acid oxidation of the amino acid has been described. The

method is rapid and gives highly reproducible results. The isoleucine content of acid and enzymic hydrolyzates of a number of biological products are reported.

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The Relation of Boron to Certain Plant Oxidases

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INTRODUCTION

Although the essential nature of boron for normal growth of higher plants is unquestioned, it has not been possible to attribute to it any specific metabolic function. The fact that boron is required in very small amounts suggests that it may function, as do certain metallic elements required at similar levels, through interaction with, or as a component of, an enzyme system.

There is some indirect evidence indicating that boron may act in an inhibitory manner on the polyphenol oxidase system of plants. Herzinger (1) reported a retarding effect of boron on the darkening of potato and beet root slices during a 48 hour period and attributed this decrease to an inhibition of the polyphenol oxidase system. Winfield (2, 3), however, was unable consistently to confirm these observations and attributed the erratic results to inhibition of bacterial growth by boric acid. Inconclusive results were also obtained when boron was added to crude preparations of potato polyphenol oxidase acting on various substrates. In a few instances, an inhibitory effect of added boric acid was observed. It has also been reported (4) that potatoes produced in boron-deficient culture showed a darkening of the internal parenchymatous tissue, which was attributed to the formation of melanin type pigments. Since the observations reported here were completed, Reed (5) has presented data based on histochemical examination of normal and boron-deficient plants indicating higher concentration of certain oxidases in the latter.

EXPERIMENTAL

Tomato (*Lycopersicon esculentum* Mill., var. John Baer), soybean (*Glycine max.* Murr, var. Biloxi), tobacco (*Nicotiana tabacum* L., var. Maryland Mammoth), and cabbage (*Brassica capitata* L., unknown variety) were employed in this study. Seeds of soybean, tomato and tobacco were sprouted and seedlings transplanted to silica sand contained in 10 inch varnished clay pots. Cabbage was obtained from field cul-

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ture. The nutrient solution employed was essentially the same as that previously described (6). Boron, when present was added at 0.5 p.p.m. Normal greenhouse conditions for summer months existed, except that photoperiods were adjusted with incandescent lamps so that the photoperiodic sensitive plants were maintained in a continuously vegetative state.

The Warburg constant volume micro respirometer was used for all measurements of gas exchange. Boron was determined by a previously described modification (7) of the quinalizarin procedure of Berger and Truog (8). Nitrogen was determined by a semi-micro modification of the Kjeldahl procedure.

Oxygen Consumption of Tissue Homogenates from Normal and Boron-Deficient Leaves

Although the use of tissue homogenates has gained wide acceptance in studies with animal tissues, the extension of this technique to plant material has been limited. It is possible, using the Potter and Elvehjem (9) apparatus, to produce leaf tissue homogenates which maintain a rapid rate of oxygen consumption for several hours. Such preparations were used to compare the rates of oxygen uptake, without added substrates, of normal and boron-deficient tissues. For uniformity, usually the second or third apical leaves were used during these studies. Samples of deficient tissue were from plants which were showing typical boron deficiency symptoms—necrosis of the apical meristematic tissue and abnormal leaf development. Homogenates were prepared by grinding the leaf blades in twice their weight of chilled, pH 6.0, 0.1 M phosphate buffer. Microscopic examination revealed the presence of numerous intact cells. The homogenate was further diluted with buffer and pipetted into the main cup of the Warburg flasks. The $Q_{O_2}(N)$ (μl. oxygen uptake/mg. nitrogen/hr.) of various homogenates of normal and deficient tissue are presented in Table I. A more rapid rate of oxygen consumption in boron-deficient tissues was observed in all experiments. In three of the four presented, significant reduction in $Q_{O_2}(N)$ occurred in the presence of 0.01 M or 0.1 M borate. These concentrations are greatly in excess of the boron concentration of approximately 0.001 M found in fresh tissue.

Oxygen Consumption of Chloroplast Suspensions from Normal and Boron-Deficient Leaves

As an extension of the homogenization technique, which presented certain mechanical difficulties in disrupting coarse conductive tissue, oxygen consumption of cell-free extracts containing a portion of the chloroplasts was examined. It was found that the $Q_{O_2}(N)$ of such preparations was approximately one-half that obtained with whole tissue homogenates. These chloroplast suspensions were obtained essentially by the method of Granick (10). Centrifugation at a force of 400 g displaced all cells, cell fragments, and starch granules, leaving a major portion of the chloroplasts in suspension. Values for the oxygen consumption without added substrates by several preparations of normal and boron-deficient tomato and soybean plants are given in Table II. Again, a more rapid rate of oxygen consumption was found in preparations from tissues suffering from boron deprivation. In Expt. No. 1, boron determinations showed that the chloroplast suspension from normal plants contained 2.1 γ of boron/ml.

TABLE I

Comparative Rates of Oxygen Consumption by Leaf Tissue Homogenates of Normal and Boron-Deficient Tomato Plants without Added Substrates

The main chamber of the flask contained 2 ml. of normal or boron-deficient leaf tissue homogenate, and 1 ml. of pH 6.0, 0.1 M KH_2PO_4-Na_2HPO_4 buffer, or 1 ml. of H_3BO_3 in 0.1 M phosphate buffer adjusted to pH 6.0. The center well contained 0.15 ml. 20% KOH and a wick to absorb CO_2. Temperature, 37°C. Reaction time, 60 minutes. Gas phase, air.

Experiment no.	Tissue and adjuncts	$Q_{O_2}(N)$[a]	
		Normal	Boron-deficient
1	Tomato + HOH	29	60
	Tomato + 0.01 M[b] H_3BO_3	30	43
	Tomato + 0.001 M H_3BO_3	30	55
2	Tomato + HOH	22	43
	Tomato + 0.01 M H_3BO_3	18	39
	Tomato + 0.001 M H_3BO_3	21	42
3	Tomato + HOH	31	41
	Tomato + 0.1 M H_3BO_3	27	42
	Tomato + 0.01 M H_3BO_3	26	45
	Tomato + 0.001 M H_3BO_3	24	41
4	Tomato + HOH	18	38
	Tomato + 0.1 M H_3BO_3	18	31

[a] $Q_{O_2}(N)$ equals microliters O_2 uptake per mg. N per hour.
[b] Final concentration.

chloroplast suspension compared with 1.2 γ for the preparation from deficient leaf tissue. Similar results were found with soybean. This would account for approximately one-half of the boron content of the original tissue. Addition of boric acid to these preparations did not reduce the rate of oxygen consumption of boron-deficient leaf preparations to the normal level, even when boric acid was added at a concentration of 0.1 M.

Effect of Boron Deficiency on Enzyme Activity

The observed increase in oxygen consumption, without added substrate, of boron-deficient plants made an investigation of specific enzyme systems desirable. The action of cell- and chloroplast-free preparations on lactic acid, glycolic acid, and dihydroxyphenyl-L-

TABLE II

Comparative Rates of Oxygen Consumption by Chloroplast Suspensions from Leaf Tissue of Normal and Boron-Deficient Tomato and Soybean Plants; No Substrate Added

The main chamber of the flask contained 2 ml. of chloroplast suspension from normal or boron-deficient leaf tissue, and 1 ml. of pH 6.0, 0.1 M KH_2PO_4-Na_2HPO_4, or 1 ml. of H_3BO_3 solution in 0.1 M phosphate buffer adjusted to pH 6.0. The center well contained 0.15 ml. 20% KOH solution and a wick to absorb CO_2. Temperature 37°C. Gas phase, air. Reaction time, 60 minutes.

Experiment no.	Tissue and adjuncts	$Q_{O_2}(N)$[a]	
		Normal	Boron-deficient
1	Tomato + HOH	9	19
	Tomato + 0.1 M[b] H_3BO_3	8	12
	Tomato + 0.01 M H_3BO_3	10	22
	Tomato + 0.001 M H_3BO_3	10	19
2	Tomato + HOH	9	24
	Tomato + 0.1 M H_3BO_3	9	20
3	Tomato + HOH	13	22
	Tomato + 0.1 M H_3BO_3	12	20
4	Soybean + HOH	13	23
	Soybean + 0.1 M H_3BO_3	12	20
5	Soybean + HOH	15	19
	Soybean + 0.1 M H_3BO_3	15	15

[a] $Q_{O_2}(N)$ equals μl. O_2 uptake/mg. N/hr.

[b] Final concentration.

alanine was followed. These preparations were made by grinding the tissue with pH 6.0, 0.1 M phosphate buffer and sand in a mortar until a homogeneous mass was obtained. The sap was expressed through muslin and the residue successively re-extracted. The combined extracts were adjusted to pH 8.0 and centrifuged at approximately 10,000 g in a small air driven centrifuge. Representative data on the oxidation of substrates by these preparations are given in Table III. Examination of the data shows that there is a less rapid oxidation of lactate and glycolate in the deficient tissue on a unit volume basis. This difference

TABLE III

Comparative Rates of Oxidation of Lactic and Glycolic Acids and Dihydroxyphenyl-L-Alanine (DOPA) by Cell-Free Extracts of Normal and Boron-Deficient Tomato Plants

The main chamber of the flask contained 1 ml. of cell-free extract from normal or boron-deficient leaf tissue, 1 ml. of distilled water, or 0.03 M boric acid solution neutralized to pH 8.0, and 1 ml. of substrate neutralized to pH 8.0. The center well contained 0.15 ml. of 20% KOH and a wick to absorb CO_2. Temperature, 37°C. Gas phase, air. Reaction time, 60 minutes.

Experiment no.	Tissue and adjuncts	Substrate and final concentration	Oxygen consumed			
			Normal		Boron-deficient	
			$\mu l./ml./hr.$	$Q_{O_2}(N)$	$\mu l./ml./hr.$	$Q_{O_2}(N)$
1	Tomato+HOH	0.0033 M DOPA	166	108	232	235
	Tomato+0.01 M^a H_3BO_3	0.0033 M DOPA	102	66	178	181
	Tomato+HOH	0.0066 M lactate	29	19	18	18
	Tomato+HOH	0.0066 M glycolate	51	34	40	41
2	Tomato+HOH		20	8	15	13
	Tomato+HOH	0.0033 M DOPA	150	67	216	196
	Tomato+HOH	0.0066 M lactate	60	27	28	26
	Tomato+HOH	0.0066 M glycolate	176	79	73	67

[a] Final concentration.

largely disappears when referred to equivalence in terms of nitrogen. The oxidation of dihydroxyphenyl-L-alanine, however, is increased by about 45% on a volume basis, and 110% on a nitrogen basis in the deficient tissue compared with that receiving boron. Boric acid at 0.01 M concentration was found in one instance to inhibit the normal and boron-deficient tissue by 40 and 23%, respectively.

Effect of Boric Acid on Various Oxidative Enzymes

In view of the enhanced endogenous oxygen consumption and polyphenoloxidase activity associated with boron deficiency, the effect of high concentrations of this element was of interest. The following systems were studied: glycolic and lactic acid oxidation by cell-free extracts of tobacco, dihydroxyphenyl-L-alanine oxidation by extracts of soybean, and ascorbic acid oxidation by extracts of cabbage. All of these had previously been shown to give rapid and reproducible rates

of oxidation with the cell- and chloroplast-free preparation previously described. All of these substrates also contain configurations which might conceivably form a complex with boric acid. The data obtained in representative trials are presented in Table IV. No inhibition of the

TABLE IV

Effect of Boric Acid on the Oxidation of Ascorbic Acid, Lactic Acid, Glycolic Acid, and Dihydroxyphenyl-L-Alanine by Cell-Free Extracts of Cabbage, Tobacco, and Soybean

The oxygen uptake corrected for enzyme blank is expressed as $Q_{O_2}(N)$. Flasks contained 1 ml. of cell-free extract of leaf tissue, 1 ml. of water or boric acid, final concentration indicated, 1 ml. of substrate to give final concentration of 0.0066 M dissolved in 0.1 M KH_2PO_4-Na_2HPO_4 buffer adjusted to pH 6.0. Blank contained 1 ml. of buffer. Center well contained 0.15 ml. of 20% KOH and a wick to absorb CO_2. Final volume, 3.15 ml.; gas phase, air; 30°C. for ascorbic acid, 37°C. for others; pH, 6.0.

Experiment no.	Tissue and adjuncts	Substrate	Oxygen consumed	Inhibition
			$Q_{O_2}(N)$	*per cent*
1	Cabbage + HOH	$M/150$ Ascorbic acid	126	9
	Cabbage + M H_3BO_3		108	14
	Cabbage + 0.1 M H_3BO_3		132	0
	Cabbage + 0.01 M H_3BO_3		123	2
	Cabbage + 0.001 M H_3BO_3		129	0
2	Tobacco + HOH	$M/150$ Glycolic acid	140	0
	Tobacco + M H_3BO_3		76	46
	Tobacco + 0.1 M H_3BO_3		105	25
	Tobacco + 0.01 M H_3BO_3		110	21
	Tobacco + 0.001 M H_3BO_3		120	14
3	Tobacco + HOH	$M/150$ Lactic acid	25	0
	Tobacco + M H_3BO_3		1	96
	Tobacco + 0.1 M H_3BO_3		16	36
	Tobacco + 0.01 M H_3BO_3		25	0
	Tobacco + 0.001 M H_3BO_3		27	0
4	Soybean + HOH	$M/150$ Dihydroxyphenyl-L-alanine	86	0
	Soybean + M H_3BO_3		9	90
	Soybean + 0.1 M H_3BO_3		24	72
	Soybean + 0.01 M H_3BO_3		67	22
	Soybean + 0.001 M H_3BO_3		75	13

ascorbic acid oxidase was apparent until the highest concentration of boric acid was used. At the pH employed (6.0), this molar solution of boric acid is essentially saturated. Almost complete inhibition of lactic acid oxidation was obtained with a similar concentration, and, with 0.1 M concentration, inhibitions from 10 to 36% were observed in different runs. No measurable inhibition was obtained with lower concentrations. The rate of glycolic acid oxidation was reduced 25% by 0.1 M boric acid. The oxidation of dihydroxyphenyl-L-alanine, however, showed significant inhibition with 0.01 M borate—18, 25, and 34% in three different trials, and some inhibition even at 0.001 M concentration. The inhibitory effect of boric acid was also evidenced by the less rapid formation of the "melanin" pigment, which results from the oxidation of dihydroxyphenyl-L-alanine.

DISCUSSION

Examination of the foregoing data indicates that one of the defects occurring in the absence of adequate supplies of boron may be an abnormal respiratory mechanism. An enhanced oxygen consumption was noted in both whole tissue homogenates and chloroplast suspensions from boron-deficient plants. A marked increase in the polyphenoloxidase activity was also observed. This enhanced rate of oxidation may be one of the causes of the decreased carbohydrate content of apical tissues of boron-deficient plants (11).

It is always difficult to compare *in vitro* and *in vivo* concentrations of substances, for within the cell a particular compound may be highly concentrated in a limited area. With cell-free preparations it may be necessary to reproduce this high local concentration in the entire medium to achieve the activity apparent in the intact cell. Since the total amount of a compound in a tissue is often a poor criterion for predicting its *in vivo* activity from *in vitro* observations, conclusions must be drawn from a comparison of tissues normal and deficient in the compound in question. These difficulties are not unusually serious in the present studies, for boric acid at the 0.001 M level noticeably inhibited polyphenoloxidase activity and concentrations of boron approaching this are often observed in normal tissues.

Normal tissues usually contain from 5 to 10 times more boron than deficient ones. This high boron content conceivably might inhibit the polyphenoloxidase system in normal tissues, whereas in boron-deficient

tissues this enzyme might have enhanced activity as a terminal oxidase. The general insensitivity of the oxygen consumption, without added substrate, of plant tissue homogenates and chloroplast suspensions to inhibition by borate concentrations which markedly inhibit polyphenoloxidase activity suggests that the polyphenoloxidase system is not the predominant normal pathway for the transfer of hydrogen to molecular oxygen. Furthermore, as can be seen in Tables I and II, there is a general tendency for the oxygen consumption of boron-deficient plants without added substrates to be more sensitive to borate than normal tissues, indicating that this enhanced respiration may be due, in part, to increased polyphenoloxidase activity. Bonner and Wildman (12) have recently suggested that polyphenoloxidase functions as the principal terminal oxidase in spinach leaves. They base their conclusions largely upon the reduction in oxygen consumption of intact leaf blade sections following infiltration with *o*-nitrophenol. This inhibitor is being tested to ascertain the effects of boron in concentrations shown to strongly inhibit polyphenoloxidase in cell-free extracts of plant tissue.

SUMMARY

Techniques are described for the preparation of homogenates of leaf tissue and cell-free extracts retaining oxidative activity without added substrate. Employing these preparations, an enhanced rate of oxygen consumption, without addition of substrate, has been demonstrated in boron-deficient leaf tissue. Boron-deficient tissue has also been shown to have a more active polyphenoloxidase than that present in normal tissue. The oxidation of dihydroxyphenyl-L-alanine by tobacco, tomato, and soybean polyphenoloxidase has been shown to be inhibited by 0.01 M borate. The oxidation of glycolic and lactic acids is affected less and ascorbic acid oxidation is not inhibited. The endogenous oxygen consumption of normal tissue preparations is likewise affected only slightly.

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The Influence of Dietary Cystine and Choline on the Total Cystine, Oxidizable Sulfhydryls, and Succinic Dehydrogenase of Rat Liver

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INTRODUCTION

Previous investigations (1) have shown that the feeding of diets containing cystine causes the development of such pathological changes as fatty livers, hepatic necrosis and cirrhosis, and renal hemorrhage. It has also been established that choline and methionine prevent or diminish these pathological changes (2). Earle and Victor (3) drew attention to the fact that liver lesions were proportional to the level of cystine in the diet, and that choline prevented the effects of small doses of cystine but not of large ones. Gyorgy and Goldblatt (4) have since proposed that the cystine to choline ratio may be considered a leading etiological factor in the prevention or production of liver cirrhosis. Increased intake of choline had a beneficial effect, whereas greater cystine intake caused cirrhosis.

Welch, Irving and Best (5) determined oxygen uptake of liver slices from rats to which a low choline diet was fed. After eating this diet for three weeks, the animals had developed fatty livers. These investigators concluded that the decrease in oxygen consumption, which resulted from feeding low choline diets, was not proportional to the development of fatty livers but exceeded it.

Potter and DuBois (6) found that cystine and cysteine inhibited the succinic dehydrogenase activity of rat liver tissue *in vitro*, and concluded that the inhibition was due to a reaction with sulfhydryl groups of the enzyme. In a series of studies, Ames and Elvehjem (7, 8, 9) also demonstrated that cystine inhibited succinic dehydrogenase activity *in vitro*.

From the investigations mentioned above, it seemed possible that cystine or one of its metabolic products might exert some of its patho-

genic action by inhibiting succinic dehydrogenase *in vivo*, and that choline chloride might prevent this inhibition by influencing some phase of the metabolism of cystine. The present study was undertaken to measure the effect of feeding diets in which the cystine and choline chloride contents were varied, on the succinic dehydrogenase activity, oxidizable sulfhydryl compounds, and total cystine of rat livers.

EXPERIMENTAL

Diets

In this study the synthetic control diet, diet (A), contained casein (Merck edible) 18%, dextrin 50%, cerelose 15%, celluflour 2%, salt (10) 3%, lard 4%, corn oil 4%, and cod liver oil 4%. By supplementing this control diet with L-cystine and choline chloride at the expense of dextrin, the following experimental diets were prepared and will be designated hereafter by the assigned letter: (B) dextrin 45%, L-cystine 5%, and choline chloride 0.6%; (C) dextrin 45%, L-cystine 5%; (D) dextrin 38%, L-cystine 10%, and choline chloride 2.4%; (E) dextrin 40%, L-cystine 10%. (In all these diets, the other components were in the quantities stated above.) Purina Laboratory Chow was also used as a control diet in some cases. A vitamin supplement containing thiamine hydrochloride 0.25 mg., pyridoxine hydrochloride 0.25 mg., nicotinic acid 0.25 mg., riboflavin 0.5 mg., calcium pantothenate 2.5 mg., α-tocopherol 10 mg., and Wilson's 1–20 liver extract 625 mg., was added/100 g. of diet. Neither the quantity of choline in the liver extract nor in Merck edible casein was measured but was considered unimportant because of the large differences in choline content of the experimental diets.

Methods

Male and female albino rats of the Sprague-Dawley strain weighing approximately 200 g. were used in these experiments. The animals were kept in individual cages, and were given distilled water to drink. Prior to eating the experimental diets, they were maintained on Purina Laboratory Chow.

The rats were fed *ad libitum* the diets described above for varying periods. At the end of the feeding periods the animals were sacrificed by decapitation and their livers immediately removed. Part of the liver was weighed and a 5% homogenate in cold distilled water was prepared by the technique of Potter and Elvehjem (11). Succinic dehydrogenase activity of this homogenate was measured by the method of Schneider and Potter (12).

A second portion of the liver was employed for the measurement of oxidizable sulfhydryl compounds. A 10% liver homogenate was prepared, allowed to stand for 15 minutes, and centrifuged at 3,000 r.p.m. for 10 minutes. Preparation of the tissue in this manner is essentially the procedure of Greenstein (13). The analysis for oxidizable sulfhydryls in the supernatant was carried out according to the method of Anson (14), and a Coleman spectrophotometer was employed for measuring the color which was developed.

The third portion of liver used for total cystine analysis was placed in a 50–50 mixture of ethanol and ethyl ether for 12 hours. The dehydrated tissue was then extracted with ether in a Soxhlet apparatus for 24 hours. After drying in air, the sample was ground into a fine powder in a mortar. A quantity of powdered liver containing between 5 and 10 mg. of cystine was hydrolyzed with 20% hydrochloric acid for 12 hours. The cystine content of the hydrolyzate was measured by the method of Sullivan and Hess (15), employing the modifications of Evans (16) for use with the Coleman spectrophotometer.

RESULTS

Food Consumption and Growth Rate

The quantity of food consumed, and the growth rate of animals eating the experimental diets is shown in Table I. It will be noted that

TABLE I

Growth Rate and Food Consumption of Rats Eating Experimental Diets

Diet	Weight gain g./day	Food consumption g./day
A	3.5	16
B	4.5	16
C	0.5	10.0
D	−4.1	10.5
E	−4.1	10.0

animals receiving the control diet (A) showed a normal growth of 3.5 g. per day and consumed *ad libitum* 16 g. of food per day. Those animals receiving diet B exhibited a growth rate and food consumption comparable to the controls. When the other three diets (C, D, and E) were fed, there was a reduction both in food intake and growth rate.

It has been indicated by Hodge (17) that quantities of choline chloride greater than 1%, in an otherwise normal diet, inhibited the growth rate of rats, and he noted that a 10% choline chloride diet completely prevented rats from growing. No histopathological effects were observed which would be considered characteristic of choline poisoning. In the present experiments choline chloride, in a 5% cystine diet, protected against the reduction in growth rate observed when a 5% cystine diet, which contained no added choline, was fed. Loss in weight of animals receiving diet E was 4.1 g. per day, and a similar weight decrease was noted when a high quantity of choline chloride was incorporated into the diet (diet D). It is possible that the high level of choline chloride in diet D caused some inhibition of growth.

Succinic Dehydrogenase

The effect of feeding the experimental diets described above for varying periods of time on succinic dehydrogenase of rat liver is shown in Col. 2 of Table II. The data are expressed as mm.[3] of oxygen consumed/mg. of dry tissue/hr. corrected to 10%

TABLE II

*Determination of Succinic Dehydrogenase Activity, Oxidizable Sulfhydryls,
and Total Cystine of Rats Fed Various Diets*

Diet and feeding period	Succinic dehydrogenase Q_{O_2} (corrected to 10% fat)	Sulfhydryls mg. cysteine/ g. dry liver homogenate (corrected to 10% fat)	Cystine mg./g. dry fat-free liver	Number of animals and determinations	Per cent ether-extractable material from dried liver
Purina control	86.7 ± 19.3^a	12.8 ± 0.2	6.2 ± 0.12	9	10(9–11)
A 2–30 Days	83.4 ± 2.9	11.9 ± 0.23	6.2 ± 0.27	20	10(8–11)
B 2–30 Days	69.6 ± 2.3	11.2 ± 0.32	6.1 ± 0.1	16	11(9–13)
2–20 Days	69.2 ± 2.6	14.2 ± 0.55^b	6.6 ± 0.11	12	13(10–15)
C 30 Days	58.4 ± 2.5^b	16.1 ± 0.2^b	6.8 ± 0.22	4	20(14–22)
D 10 Days	63.6 ± 8.2^b	13.5 ± 0.47	6.7 ± 0.43	4	16(13 18)
E 10 Days	55.0 ± 4.8^b	17.9 ± 2.3^b	7.1 ± 0.1^b	4	27(23–32)

[a] Plus and minus values indicate the standard error of the arithmetic mean.

[b] Values which showed a significant difference from the control as measured by "Student's" t test ($P = 0.01$).

fat per unit of dry weight. It will be noted in Col. 6 of Table II that fatty livers often developed in animals eating the high cystine experimental diets. These variations in fat content influence the Q_{O_2} values based on succinic dehydrogenase activity per unit dry weight of tissue. To eliminate the influence of variations in fat content of the tissues, all values were corrected to the normal of 10% fat per unit dry weight.

It will be seen that there was some variation in the succinic dehydrogenase activity of normal liver, but, in agreement with other investigators, the average Q_{O_2} values for animals consuming the control diet was 83.4 and for rats receiving Purina Chow was 86.7.

The data indicate a definite decrease in succinic dehydrogenase activity in the livers of animals eating high cystine diets. Rats which had eaten diet B (a 5% cystine diet supplemented with 0.6% choline) for periods varying from 2 to 30 days showed liver succinic dehydrogenase activity little different from the normal. The average Q_{O_2} for a series of measurements made after 2, 5, 10, 20, and 30 day feeding periods was 69.6. Many of the values fell within the normal range, and there appeared to be no definite relationship between succinic dehydrogenase activity and the length of the feeding period. The fat content of the livers of these animals was also normal.

Rats which were fed diet C (a 5% cystine diet which contained no added choline) presented a somewhat different picture. After feeding such a diet for periods varying from 2 to 20 days, succinic dehydrogenase activity was only slightly below the control value. However, increasing the feeding period to 30 days, resulted in a significant decrease in activity as indicated by an average succinic dehydrogenase Q_{O_2} of 58.4, which is only 69% of the control value. It was noted that after 30 days excessive fat deposition had occurred in the liver as a result of eating the high cystine diet.

That a decrease in succinic dehydrogenase activity follows the extent of liver damage caused by cystine is well illustrated by the results of feeding two 10% cystine diets. Of 4 animals eating diet D (a 10% cystine diet containing 2.4% choline) all showed some excess liver fat deposition. However, none of the animals in this group died during the 10-day feeding period. The average succinic dehydrogenase activity of the livers of these animals was 63.6, a Q_{O_2} which is 76% of normal activity. Since Hodge (17) has shown no pathological condition in the liver resulting from choline alone, it seemed that the reduction in succinic dehydrogenase did not result from the high choline chloride level of the diet.

Of 4 animals eating diet E (a 10% cystine diet containing no added choline) for 10 days, all deposited a large amount of fat in their livers. One animal fed this diet for 10 days appeared to have a normal liver and therefore was not included with the data presented in Table II. It is interesting to note that of 8 rats begun on this diet, only 5 survived the 10-day feeding period. As a result of feeding the diet, succinic dehydrogenase activity was greatly lowered as is indicated by a Q_{O_2} of 55.0. This activity is 65% of the control value, a significant difference.

It appears that, when animals are fed high cystine diets, succinic dehydrogenase activity decreases in proportion to liver damage. It appears also that choline protects slightly against the lowering of succinic dehydrogenase activity by cystine. It is apparent, however, that the decrease in enzyme activity is not a result of dilution of active tissue with fat, but is rather an actual reduction in succinic dehydrogenase activity in the tissue.

Oxidizable Sulfhydryl Compounds

The data obtained from the determination of sulfhydryl groups in the livers of rats fed high cystine diets for various periods of time is presented in Col. 3 of Table II. The results are expressed as mg. of cysteine/g. of dried, centrifuged liver homogenate, and again were corrected to 10% liver fat to compensate for dilution of active tissue. Although no attempt was made in this study to determine specifically the structures of the compounds containing sulfhydryl groups, it has been shown that sulhydryl compounds were actually being measured in these experiments by the analytical method employed. Mason (18) has demonstrated that formaldehyde reacts with compounds contain-

ing sulfhydryl groups rendering them unoxidizable by ferricyanide. Formaldehyde does not destroy the ability of ascorbic acid, uric acid, or other compounds, which might cause erroneous results in this study, to reduce ferricyanide. When a tissue homogenate was treated with formaldehyde, no development of color resulted on application of the analytical method, indicating that interfering oxidizable substances were not present.

The results indicated that the normal quantity of sulfhydryl groups in rat liver expressed as cysteine is 11.9 mg./g. of dry, centrifuged liver homogenate. This value was not altered during a 30-day control feeding period; nor was there any difference in the quantity of sulfhydryl groups in the livers of animals receiving Purina Laboratory Chow.

Animals eating diet B showed no significant change from normal oxidizable sulfhydryl groups of the liver. A definite rise in oxidizable sulfhydryl compounds was noted, however, when diet C, which contained no added choline, was provided. When this diet was fed during periods varying from 2 to 20 days, an increase from 11.9, the normal value, to 14.2 mg. of cysteine/g. of dry liver homogenate was found. The oxidizable sulfhydryls continued to increase during the 30 day feeding period, at the end of which the average of 4 animals was 16.1 mg. of cysteine/g.

The results obtained after rats had eaten the two 10% cystine diets illustrate clearly the fact that choline is necessary to maintain a normal quantity of liver sulfhydryls. Animals eating diet D for 10 days had 13.5 mg. cysteine/g. of dry liver homogenate, which is only slightly above the control and did not prove to be a significant difference. Those rats receiving diet E showed a definite increase in liver sulfhydryls. The average value for 4 animals was 17.9 mg. cysteine/g. of dried, centrifuged liver homogenate. It appears that high cystine diets cause a rise in liver oxidizable sulfhydryl compounds but that this rise can be almost completely prevented by increasing dietary choline.

Total Cystine

The total liver cystine values obtained by feeding high cystine diets are presented in Col. 4 of Table II. These data are expressed as mg. of cystine/g. of dry defatted liver.

The data indicate that the normal total cystine is 6.2 mg./g. of dry defatted liver. There was no significant difference between the total liver cystine of animals receiving the control diet and those eating

Purina Laboratory Chow. The only significant difference in cystine content of the livers of rats fed the various diets resulted after feeding the 10% cystine diet which contained no added choline chloride (diet E). It will be noted that in this instance the total liver cystine was 7.1 mg. cystine/g. of dry defatted liver. When the other high cystine diets were fed, there appeared to be an increase in liver cystine which indicated a tendency for liver cystine to rise. It may be concluded that the addition of choline to high cystine diets prevented, to some extent, a rise in liver cystine.

Discussion

Axelrod, Potter and Elvehjem (19) indicated that an adequate daily intake of riboflavin was necessary for optimum succinic dehydrogenase activity. It was also shown by Rosenthal (20) and by Axelrod, Swingle and Elvehjem (21) that partial starvation (3–4 g. food per day) had little effect upon succinic dehydrogenase activity (based on dry weight of liver homogenate) as long as the riboflavin content of the diet was adequate.

Recently, in contradiction to these data, Elson (22) has stated that succinic dehydrogenase activity is directly related to the protein content of the diet. However, Czaczkes and Guggenheim (23) showed that as the per cent protein in the diet decreases the requirements for riboflavin increases. They state that one can cause a riboflavin deficiency by lowering the protein content of the diet. It is indicated, therefore, that, when food consumption is lowered, the riboflavin content of the diet must be kept adequate if optimum enzyme activity is to be maintained.

In our experiments riboflavin intake in all cases was equal to or greater than the quantity suggested by Axelrod *et al.* (19) to be adequate (100 γ per day).

The present study indicates that the feeding of high cystine diets to rats caused a decrease in succinic dehydrogenase activity of liver tissue. The addition of choline to these diets appeared in a small measure to prevent the decrease in enzyme activity. Correction of the succinic dehydrogenase Q_{O_2} for excessive fat deposition indicated that the decrease in enzyme activity greatly exceeded the dilution of active tissue by fat. The feeding of high cystine diets caused, as well, a marked rise in the quantity of liver-oxidizable sulfhydryl compounds which was accompanied by a somewhat smaller rise in total liver cystine. Increases

in both sulfhydryls and cystine were in some measure prevented by dietary choline.

The present study is in agreement with the findings of Welch *et al.* (5) who showed that low choline diets caused a decrease in oxygen uptake of liver slices of rats. These investigators measured the oxygen uptake of liver slices incubated with glycerol phosphate, and it is probable that the decrease in oxygen consumption, during the oxidation of glycerol phosphate was due to a decrease in triosephosphate dehydrogenase, an enzyme which is dependent upon sulfhydryl groups for its activity.

These *in vivo* experiments are in agreement with the *in vitro* findings of Potter and DuBois (6) and Ames and Elvehjem (7, 8, 9,) who demonstrated that cystine and cysteine inactivate succinic dehydrogenase. The present study, however, does not ascertain whether it is the increase in sulfhydryls or the rise in total cystine which is associated with the inactivation. It is indicated that a rise in titratable sulfhydryls and total cystine of liver resulting from feeding high cystine diets is accompanied by decreased succinic dehydrogenase activity. Dietary choline appears to decrease the inactivation of sulfhydryl enzymes by preventing the accumulation of sulfides and disulfides during metabolism.

SUMMARY

1. The feeding of high cystine diets containing no choline to rats resulted in a rise in oxidizable sulfhydryl compounds and total cystine which was accompanied by a reduction in succinic dehydrogenase activity.

2. The addition of choline to high cystine diets prevented the increase in quantity of liver sulfhydryls and appeared to protect against a rise in total liver cystine. Choline appeared, as well, to prevent a decrease in liver succinic dehydrogenase activity, but the differences were not statistically significant.

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Further Studies on α,α-Di(Acylamino)Propionic Acids

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INTRODUCTION

As part of a general study on dehydropeptides and related compounds (*cf*. 1–3), the investigation has been undertaken of the preparation and biological properties of α,α-di(acylamino)propionic acids, *e.g.*, $(RCONH)_2C(CH_3)COOH$ (2). On enzymatic hydrolysis, favorably constituted dehydropeptides and di(acylamino)propionic acids yield ammonia and pyruvic acid. α,α-Di(glycylamino)propionic acid is readily hydrolyzed by fresh aqueous extracts of several rat tissues to yield equivalent amounts of ammonia and pyruvic acid (2). With kidney extracts at the optimal pH of 8.0, maximum hydrolysis of the substrate yields 1 mole of ammonia and 1 mole of pyruvic acid/mole of substrate. Neither α,α-di(acetylamino)propionic acid nor α,α-di-(chloroacetylamino)propionic acid is enzymatically attacked.

In the endeavor to extend the study of this novel class of substrates, α,α-di(DL-chloropropionylamino)propionic acid (III) was prepared by the general procedure developed earlier, involving the condensation of 2 moles of DL-chloropropionitrile (I) with 1 mole of pyruvic acid (II) dissolved in chilled, concentrated sulfuric acid (2, 4). On partial amination of III, α-(DL-alanylamino)-α-(DL-chloropropionylamino)-propionic acid (IV) was obtained. Further amination of IV yielded α,α-di(DL-alanylamino)propionic acid monohydrochloride (V).

$$2CH_3CH(Cl)CN + CH_3COCOOH \xrightarrow{\quad H_2O \quad}$$
$$\underset{I}{} \quad \underset{II}{}$$

$$\underset{III}{[CH_3CH(Cl)CONH]_2C(CH_3)COOH}$$

$$\xrightarrow{\quad NH_3 \quad} \underset{IV}{[CH_3CH(NH_2)CONH][CH_3CH(Cl)CONH]C(CH_3)COOH}$$

$$\xrightarrow{\quad NH_3 \quad} \underset{V}{[CH_3CH(NH_2)CONH]_2C(CH_3)COOH \cdot HCl}$$
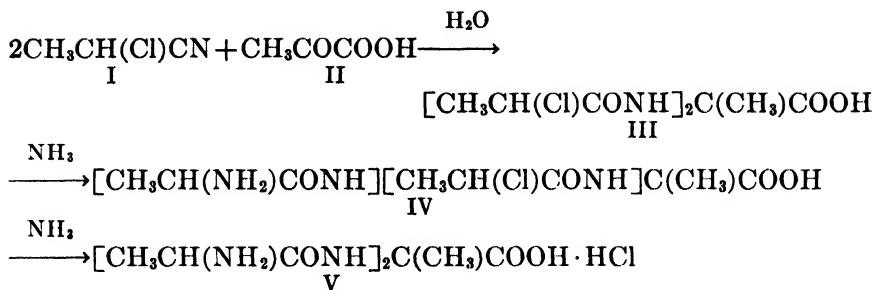
Since DL-chloropropionitrile (I) and pyruvic acid (II) were used as starting materials, products III, IV, and V are mixtures of all possible isomers. Compounds III and V possess two asymmetric carbon atoms and each may, therefore, exist in four forms. Compound IV possesses three asymmetric carbon atoms and hence may consist essentially of eight isomeric substances or four pairs of optical enantiomorphs.

The preparation of compounds III, IV, and V, and their susceptibility to enzymatic attack by aqueous extracts of rat kidney, form the bulk of this report.

EXPERIMENTAL PROCEDURE

α,α-Di(DL-chloropropionylamino)propionic Acid (III)

Forty-eight g. of freshly distilled DL-α-chloropropionitrile (5) were added dropwise over a two hour period to a solution of 22 g. of freshly distilled pyruvic acid in chilled concentrated sulfuric acid. The temperature was never allowed to rise above 15°C. The mixture was then poured in a thin stream with stirring over 4 l. of ice shavings. The product appeared in a gummy, white mass which solidified on standing overnight in the ice chest. The product was pulverized, washed thoroughly with water, and crystallized from hot water. Flat prisms. The yield was 36 g. or 50% of the theory based on the pyruvic acid used. M.p. 185°C.

Calculated for $C_9H_{14}O_4N_2Cl_2$: N, 9.7; Cl, 24.9. Found: N, 9.6; Cl, 24.8.

α-(DL-Alanylamino)-α-(DL-Chloropropionylamino)propionic Acid (IV)

Ten g. of di(chloropropionylamino)propionic acid were dissolved in 100 cc. of 28% aqueous ammonia. After standing for 3 days at 30°C., the solution was evaporated in vacuo to a thick syrup. On treatment with an excess of absolute alcohol the product solidified to a white mass. It was twice dissolved in the minimum amount of cold water and precipitated each time with an excess of absolute alcohol. A white, semi-crystalline powder was obtained which weighed 6 g., amounting to about 50% of the theoretical yield. M.p. 207°C. with decomposition. The peptide is bound to 2 molecules of water. After drying at 78°C. and 1 mm. pressure of Hg, it loses 11.2% in weight; calculated for 2 molecules of water 11.9%. There was no measurable optical rotation

for a 0.4% solution. The peptide is easily soluble in water, insoluble in non-aqueous solvents. Its aqueous solution is slightly acid to litmus. Analysis of the dried compound is as follows:

Calculated for $C_9H_{16}O_4N_3Cl$: C, 40.6; H, 6.2; N, 15.8; Cl(total), 13.3; Cl(ionic), 0.0. Found: C, 40.1; H, 6.3; N, 15.8; Cl(total), 12.4; Cl(ionic), <1.0.

The compound is not completely pure, for it contains a trace of ionic chlorine which repeated precipitations from alcohol-water mixtures were unable to remove. The impurity, which may amount to 3–5% of the compound, is presumably α,α-di(DL-alanylamino)-propionic acid·HCl. It is not believed that this small amount of accompanying material would materially affect the enzymatic data obtained on the bulk of compound IV.

The finding that under these conditions practically only one of the chloropropionylamino residues was aminated, was somewhat unexpected. That the substance is not an equal mixture of starting material and completely aminated material follows from the analytical data, and particularly from the nearly negligible values for ionic chlorine. In order to obtain the completely aminated product, the reaction was conducted under pressure in ammonia water saturated at 0°C.

α,α-Di(DL-Alanylamino)propionic Acid HCl (V)

Two g. of α-(DL-alanylamino)-α-(-DL-chloropropionylamino)propionic acid were dissolved in 30 cc. of aqueous ammonia, saturated at 0°C. After standing in a sealed flask for 8 days at 40°C., the solution was evaporated in vacuo to a thick syrup. On treatment with an excess of absolute alcohol the product solidified to a white mass. It was thoroughly triturated with alcohol to remove ammonium chloride, then dissolved in the minimum amount of water and precipitated with an excess of absolute alcohol. A white powder was obtained which weighed 0.8 g, amounting to about 40% of the theoretical yield. M.p. 196°C. with decomposition. The compound is bound to 1 molecule of water which can be removed by drying at 78°C. and 1 mm. Hg pressure. Calculated for 1 molecule of water 5.9%; found 5.8%. No optical rotation could be observed in a 0.4% aqueous solution. The peptide is easily soluble in water, insoluble in non-aqueous solvents. Its aqueous solution is slightly acid to litmus. Analysis of the dried compound is as follows:

Calculated for $C_9H_{19}O_4N_4Cl$: C, 38.2; H, 6.7; N, 19.8; Cl(ionic), 12.5; Cl(total), 12.5. Found: C, 38.1; H, 6.4; N, 19.7; Cl(ionic), 11.7; Cl(total), 11.8.

Practically all of the total (Pregl) chlorine consists of ionic chlorine, equivalent to what would be expected for close to one mole of hydrochloric acid per mole of compound.

HCl Hydrolyzates of the Peptides

Complete acid hydrolysis of α,α-di(acetylamino)propionic acid, α,α-di(chloroacetylamino)propionic acid, and α,α-di(glycylamino)-propionic acid yields 2 moles of ammonia and 1 mole of pyruvic acid per mole of compound used (2). The ammonia presumably is derived from the two atoms of nitrogen linked to the tertiary carbon atom. The same procedure as before (2) was followed in the case of di(DL-chloropropionylamino)propionic acid, (DL-alanylamino)-(DL-chloro-propionylamino)propionic acid, and di(DL-alanylamino)propionic acid · HCl.

After boiling solutions of 25 μM of each of these three compounds in 2 N HCl under the reflux condenser for 2 hours, there was obtained, respectively, 48, 47, and 47 μM of ammonia-N together with 24, 23, and 24 μM of pyruvic acid. By means of this acid hydrolytic treatment, therefore, all of the diacylaminopropionic acids yield close to 2 moles of ammonia-N and 1 mole of pyruvic acid.

DL-Alanine amide hydrobromide (6) and DL-α-chloropropionylamide (5) yielded all of their amide nitrogen as ammonia when treated in the above fashion.

Enzymatic Studies

The digests consisted of 1 cc. of aqueous rat kidney extract equivalent to 333 mg. fresh tissue, 2 cc. of 0.15 M borate buffer at pH 8.2, and 1 cc. of substrate solution at 0.025 M. The tissue extract had been dialyzed for 2 hours at 5°C. against distilled water just prior to the digestion. Control digests consisted in substituting 1 cc. of water for the substrate solution. The substrates were entirely stable in aqueous solution and in the presence of heated tissue extracts. Ammonia and pyruvic acid which appeared in the digests were measured as heretofore (2). The incubation temperature was 37.5°C. Tissues were removed freshly from decapitated rats. The results from 1 sample of the same kind of tissue to another were reproducible to 8%. The pH of the digests at the beginning of the incubation period was 7.9–8.1, and remained within this range for several hours.

α,α-Di(DL-chloropropionylamino)propionic acid incubated for 4 hours with aqueous extracts of rat kidney yielded no measurable ammonia or pyruvic acid in the digests, and hence is presumed to be resistant to the action of enzymes extractable from this tissue. On the other hand, the partially aminated product, namely, α-(DL-alanylamino)-α-(DL-chloropropionylamino)propionic acid, as well as the completely aminated product, α,α-di(DL-alanylamino)propionic acid, is susceptible to enzyme systems in the rat kidney (Figs. 1 and 2).

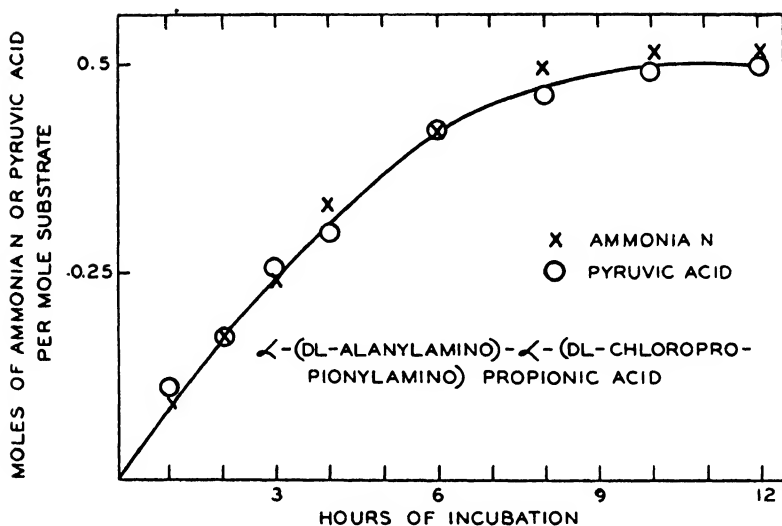α-(DL-Alanylamino)-α-(DL-chloropropionylamino)propionic acid is

FIG. 1. Time course of the enzymatic hydrolysis of α-(DL-alanylamino)-α-(DL-chloropropionylamino)propionic acid in rat kidney digests.
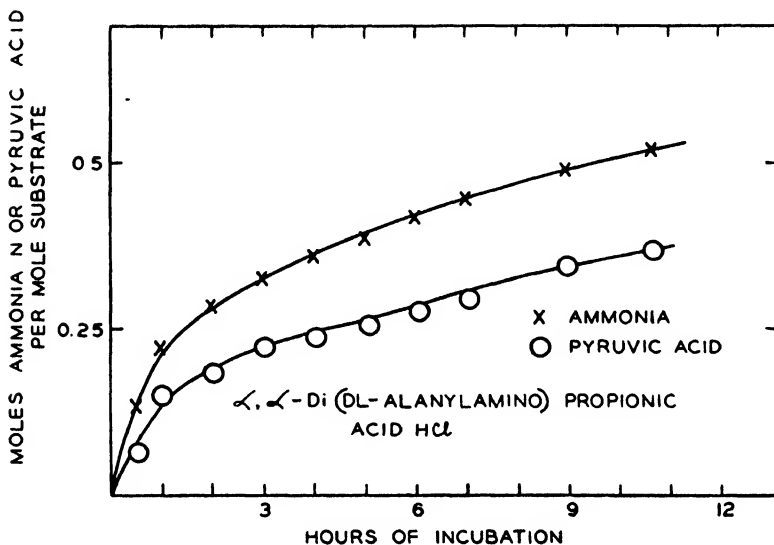
FIG. 2. Time course of the enzymatic hydrolysis of α,α-di(DL-alanylamino)-propionic acid. HCl in rat kidney digests.

enzymatically hydrolyzed to yield equivalent amounts of ammonia and pyruvic acid. At each time interval studied, the molar ratio of ammonia to pyruvic acid is close to unity, and the average ratio over the entire incubation period is 1.0. The molar ratios of ammonia N to pyruvic acid for 1, 2, 3, 4, 6, 8, 10, and 12 hours of incubation are, respectively, 0.8, 1.0, 0.9, 1.1, 1.0, 1.2, 1.1, 1.0 (Fig. 1). Under the experimental conditions employed, the maximum hydrolysis yields close to 0.5 mole of ammonia and 0.5 mole of pyruvic acid/mole of substrate (Fig. 1). Addition of fresh tissue extract to a digest already incubating for 10 hours resulted in no increase in the amount of ammonia and pyruvic acid present.

In the case of α,α-di(DL-alanylamino)propionic acid a quite different picture of enzymatic hydrolysis emerges, for with this substrate the production of ammonia and of pyruvic acid is not equimolar (Fig. 2). At each time interval, the molar ratio of ammonia-N to pyruvic acid is within the range of 1.5–1.7, the average ratio over the entire incubation period being close to 1.6. The molar ratios of ammonia N to pyruvic acid for 1, 2, 3, 4, 5, 6, 7, 9, and 11 hours of incubation are, respectively, 1.6, 1.8, 1.6, 1.7, 1.7, 1.6, 1.5, 1.5, and 1.5 (Fig. 2). Furthermore, the curves in Fig. 2 do not appear to level off within the 12 hour period of incubation, suggesting that the enzymatic reaction was not yet complete.

DL-Alanine amide at 0.05, at 0.025 and at 0.0125 M concentration, when incubated under similar conditions with rat kidney extracts, was nearly completely hydrolyzed at the amide bond in about 30 minutes of incubation; 23.8, 12.1, and 6.2 μM of ammonia-N were obtained respectively. Lengthening the period of incubation to 12 hours did not alter these values. Only one of the two optical isomers of DL-alanine amide is hydrolyzed. Incubation of di(alanylamino)-propionic acid, of (alanylamino)(chloropropionylamino)propionic acid, and of alanine amide under anaerobic conditions gave results nearly identical with those obtained under aerobic conditions. It would not appear that the products obtained were due in appreciable measure to amino acid oxidase activity. dl-α-Chloropropionylamide was not hydrolyzed in rat kidney digests whether under aerobic or anaerobic conditions.

DISCUSSION

For the production of pyruvic acid from α,α-di(acylamino)propionic acids, whether through hot acid or enzymatic action, both bonds from

the acylamino residues to the tertiary carbon atom must be broken. When hot HCl is used as hydrolytic agent, both nitrogen atoms linked with the tertiary carbon atom appear as ammonia nitrogen (2, see above). In the case of the enzymatic hydrolysis of di(glycylamino)-propionic acid only one of these nitrogen atoms appears as ammonia nitrogen (2). Substantially similar results are obtained by the enzymatic hydrolysis of (DL-alanylamino)-(DL-chloropropionylamino)propionic acid, except that, in this case, only half of the substrate is apparently susceptible to the action of extractable enzymes of the rat kidney (Fig. 1).

(DL-Alanylamino)-(DL-chloropropionylamino)propionic acid (IV) possesses three centers of asymmetry, namely, at each of the acylamino residues, and at the tertiary carbon atom, and hence yields four pairs of enantiomorphs or eight different substances. If l and d represent the two forms of asymmetry at the tertiary carbon atom without reference as to which is "natural" or "unnatural," and L and D the forms of the asymmetric carbon atoms in the acylamino residues, and NH_2 and Cl represent the α-substituted groups in the acylamino residues, then the eight substances may be represented as follows: 1) (l L NH_2, l L Cl); 2) (l L NH_2, l D Cl); 3) (l D NH_2, l L Cl); 4) (l D NH_2, l D Cl); 5) (d L Cl, d L NH_2); 6) (d D Cl, d L NH_2); 7) (d L Cl, d D NH_2), and 8) (d D Cl, d D NH_2). To account for the experimental findings of 0.5 mole of pyruvic acid produced per mole of substrate at maximum enzymatic hydrolysis, four of these eight forms must be susceptible. The choice of these probable forms must, in turn, be dependent on the fact that 0.5 mole of ammonia N is also produced per mole of substrate (Fig. 1). The nearly constant value of unity for the molar ratio of ammonia N to pyruvic acid throughout the incubation period suggests that one mole of each is produced per mole of susceptible substance.

Pyruvic acid and ammonia, however, are only the end products of the enzymatic reactions responsible for their appearance. It is probable that the enzymatic splitting of susceptible di(acylamino)propionic acids may proceed by either one of two mechanisms, namely (a) by a primary attack upon one of the peptide bonds in the acylamino residues, leading to the production of the free acyl acid and α-amino, α-acylaminopropionic acid, followed by the spontaneous hydrolysis of the latter, unstable compound to the acyl acid amide, ammonia, and pyruvic acid, or (b) by a primary attack upon one of the bonds

from an acylamino residue to the tertiary carbon, leading to the production of acyl acid amide and dehydropeptide, and followed by the hydrolysis of the latter compound, if favorably constituted, by dehydropeptidases to free acyl acid, ammonia, and pyruvic acid (2). The primary enzymatic attack upon (DL-alanylamino)-(DL-chloropropionylamino)propionic acid could conceivably lead to the production of D- and L-alanine, D- and L-alanine amide, d- and l-chloropropionic acid, d- and l-chloropropionylamide, and D- and L-alanyldehydroalanine. In this connection, the following facts must be taken into consideration: (a) neither form of dl-chloropropionylamide yields ammonia by enzymatic action (see above), (b) only one form of DL-alanine amide (presumably the L-form) (see above) and one form of DL-alanyldehydroalanine (1) (presumably the L-form) yield ammonia by enzymatic action, and (c) since nearly identical results are obtained with digests under aerobic and anaerobic conditions it is improbable that D-alanine, an excellent substrate for D-amino acid oxidase, is one of the products of the reaction.

With the above considerations in mind, it would appear that only compounds 1, 2, 5, and 6 of the eight described above satisfy the criteria of enzymatic susceptibility in respect to the products formed. Whether the initial enzymatic action is upon the peptide bond or upon the N-tertiary carbon bond, the two pairs of enantiomorphs, 1 and 5, and 2 and 6, are the only possible substances which would yield equimolar quantities of ammonia and pyruvic acid under the conditions studied. If the initial attack is upon the peptide bond, this bond must be adjacent to an L-alanine residue, and its susceptibility is independent of the configuration of the chloropropionylamino residue and of the tertiary carbon atom. If the initial attack is upon a tertiary carbon bond, the susceptibility of this bond is apparently independent of its configuration, the bond must connect to either a D- or an L-chloropropionylamino residue, while the alanylamino residue must be of the L-configuration leading to the formation of L-alanyldehydroalanine.

Conversion by further amination of α-(DL-alanylamino-α-(-DL chloropropionylamino)propionic acid to α,α-di(DL-alanylamino)propionic acid (V) results in a reduction of the number of isomeric forms to four, namely, to two *meso* forms and one pair of enantiomorphs. These substances may be represented as follows: 1) (L NH$_2$, L NH$_2$); 2) (L NH$_2$, D NH$_2$); 3) (D NH$_2$, L NH$_2$); and 4) (D NH$_2$, D NH$_2$).

Interpretation of the hydrolysis of this compound is handicapped by the fact that the hydrolysis curve does not level off during the time interval selected. Longer periods of incubation than 12 hours were not considered desirable. However, the nearly constant molar ratio of ammonia N to pyruvic acid of 1.6, together with the shape of the curves in Fig. 2, suggest that the susceptible substances are 1, and either one of the two meso forms, 2 or 3. Whether the initial enzymatic reaction is on one of the peptide bonds in an L-alanylamino residue, or on an N-tertiary carbon bond, the hydrolysis of substance 1 would yield two moles of ammonia N to one mole of pyruvic acid, while the susceptible *meso* form would yield one mole of ammonia N to one mole of pyruvic acid. Provided that the rates of hydrolysis of these susceptible forms were approximately equal, a nearly constant molar ratio of ammonia N to pyruvic acid of 1.5 throughout the incubation period should be achieved. The experimentally observed average value of 1.6 is not far from this theoretical value.

α,α-Di(glycylamino)propionic acid is related to glycyldehydroalanine insofar as the latter may be considered to be a condensation product of one mole of glycine amide with pyruvic acid, whereas the former may be considered to be a condensation product of two moles of glycine amide with pyruvic acid (*cf.* 2). Similarly, α,α-di(DL-alanylamino)propionic acid may be considered to be a product of the interaction of two, DL-alanyldehydroalanine a product of the interaction of one, moles of DL-alanine amide with pyruvic acid (*cf.* 1). All of these compounds are susceptible to enzymatic hydrolysis, yielding ammonia and pyruvic acid, the rates of hydrolysis being considerably greater for the dehydropeptides than for the di(acylamino)propionic acids (*cf.* 1). On the other hand, although the acetyl-, chloroacetyl-, and α-chloropropionyl-dehydropeptides are readily hydrolyzed by enzyme systems in the kidney (1), the corresponding di(acetylamino)-, di(chloroacetylamino)-, and di(α-chloropropionyl-amino)propionic acids are resistant to enzymatic hydrolysis (2). For enzymatic susceptibility, the di(acylamino)propionic acids require the presence of an α-amino group in at least one of the two acylamino residues.

The enzymatic hydrolysis of favorably constituted di(acylamino)-propionic acids is novel in the field of peptide metabolism. Taken together with the appreciable effect of pyruvic acid on the desamidation of glutamine and asparagine (3, 4), and with the widespread and active character of the dehydropeptidases, it is further suggestive of the possible interaction of amino acid amides and α-keto acids in animal tissues. The present study, and that earlier reported (2), may be considered as the beginning of a series of investigations on this class of substrates. So far, these studies have involved the action of tissue

extracts upon substrates which are mixtures of optical enantiomorphs, and we are under no illusion as to the difficulties in the way of an unequivocal interpretation of the phenomena. For a more exact understanding of the mode, or modes, of enzymatic degradation two lines of approach are contemplated, (1) the preparation of substrates of optical purity and individuality, and (2) the isolation and characterization of the enzymes involved.

SUMMARY

α,α-Di(DL-chloropropionylamino)propionic acid was prepared by condensing two moles of dl-chloropropionitrile with one mole of pyruvic acid in chilled, concentrated sulfuric acid. On partial amination, α-(DL-alanylamino)-α-(DL-chloropropionylamino)propionic acid was obtained, which, on further amination, yielded α,α-di(DL-alanylamino)-propionic acid · HCl.

The susceptibility to enzymatic hydrolysis of the three compounds was determined in rat kidney digests by the ammonia and pyruvic acid produced. Di(chloropropionylamino)propionic acid was completely resistant. (Alanylamino)(chloropropionylamino)propionic acid yielded a maximum of 0.5 mole of ammonia N and of 0.5 mole of pyruvic acid per mole substrate, and at each time interval during the incubation period the molar ratio of these products was close to unity. Di-(alanylamino)propionic acid yielded ammonia N and pyruvic acid in digests with rat kidney at a nearly constant molar ratio of 1.6.

The optically isomeric forms of the substrates are described and are considered in the interpretation of the phenomena observed.

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The Influence of Oxygen Tension upon the Respiration of Rat Kidney Slices [1]

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INTRODUCTION

During the course of other experiments on rat kidney slices it became apparent that the respiration of this tissue is significantly influenced by the tension of oxygen in the gas phase. The controversy
over the significance of similar observations by other workers led us to
make an independent study of this problem. The contention that
tissue respiration is independent of oxygen tension is based principally
upon experiments with unicellular organisms. Incident to the experiments on the effect of variations in oxygen tension we have compared
glucose and lactate as sustaining substrates for rat kidney respiration.

METHODS

Tissues from young white rats weighing between 50 and 150 g. were used. The rats
were anesthetized with pentobarbital sodium and killed by opening the chest. The
kidneys were removed immediately and placed on ice. Slices were made by the method
of Deutsch (6) as modified by Cohen (3). The slices were placed briefly in a small
volume of cold oxygenated Krebs' solution and subsequently removed to a white
petri dish for selection. They were then transferred to 15 cc. Warburg vessels in which
oxygen uptake was measured by the direct method. Krebs' phosphate-saline solution

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(14) was employed as medium. The average elapsed time from the death of the animal to the first reading of the manometers was less than 1 hr. The experiments were conducted at a temperature of 37°C. The duration of the experiments was usually 4 hours. At the end of this time the tissues were removed from the flasks, dried on watch crystals at 100–110°C. for 1.5-2 hours and weighed. The oxygen consumption is expressed in the conventional terms of mm.³ of oxygen N.T.P./mg. dry weight/hr. (Q_{O_2}).

The final dry weight of the tissues was used rather than the initial dry weight calculated from the initial wet weight. This is not an entirely satisfactory procedure because of the loss of weight of the tissue during the course of the experiment (2, 4). We have found that the recovery of tissue, as determined by measuring wet weights before and after shaking, is not more than $63 \pm 7\%$ when thin slices are used. Consequently, the Q_{O_2} values reported in these experiments are higher than the values that would have been obtained if the calculations had been based upon initial dry weight. On the other hand, the measurements of the initial wet weight are variable and are influenced by the imbibition of water during slicing and selection of tissues (19) and by the amount of water blotted from the tissues before they are weighed. Sperry and Brand found that about 85% of the total water taken up by liver tissues was imbibed within the first 45 minutes of soaking. We have found that the imbibition of water by liver slices immersed in Krebs' solution, which has been described by Sperry and Brand, also occurs in rat kidney slices. In a small series of experiments it was found that fresh slices of kidney contained $76 \pm 3\%$ water. If the slices were allowed to stand in Krebs' solution for 30–45 minutes and were then blotted lightly with filter paper moistened with Krebs' solution, the water content was $79 \pm 3\%$. It is, therefore, apparent that water is taken up, and in our calculations we have employed the average wet weight/dry weight ratio found on the slices, where the wet weight was determined after the preliminary soaking in Krebs' solution. This figure (corresponding to 79% water) is 4.7.

Since the thickness of the slices is an important factor in the consideration of the influence of oxygen tension upon respiration, we have attempted to estimate the thickness as accurately as possible in these experiments. It was found possible to measure the thickness very conveniently with a calibrated microscope. By placing the slice to be measured on a piece of moist filter paper on a microscope slide and focusing the microscope upon the uppermost fibers of the filter paper with the coarse adjustment (the fine adjustment set at zero), the thickness of the slice can be obtained by noting the number of divisions through which the fine adjustment must be moved to focus on the top cells of the slice. This method was checked against that employing the surface area and wet weight (or volume) of the slice. The results are in excellent agreement. The microscope method is more rapid and makes possible the estimation of the variations of thickness in different parts of the slice. In most of the experiments with air, we first determined the maximal thickness of one or two slices which were used as standards for selecting experimental slices. The maximal thickness of the slices used in nearly all experiments with low oxygen tensions was 0.18–0.2 mm. Fuhrman and Field (7) have shown that excessively thin liver slices have a lower respiration than thicker slices. They attribute this difference to a greater degree of damaged cells in the thinner slices, but, as Boyland and Boyland (1) pointed out, the greater diffusion of substrate from the thinner slices may also be a factor in lowering

their respiration. We found that the thin slices have a smaller Q_{O_2} than thicker ones, although we did not observe the extreme differences noted by Fuhrman and Field, using liver slices. So that the differences in respiration with different oxygen tensions might not be attributable to varying degrees of cell damage, we have used slices of about the same thickness in all flasks during a given experiment. To exclude the possibility that some of the slices might thicken during the experiment, we have determined the thickness of slices before and after shaking. No significant gain or loss of thickness was observed.

I. EFFECT OF THE ADDITION OF LACTATE AND GLUCOSE ON RESPIRATION

A series of experiments was performed in which varying concentrations of lactate were added to the medium; the results are shown in Fig. 1, where Q_{O_2} for the first hour of respiration is plotted against the
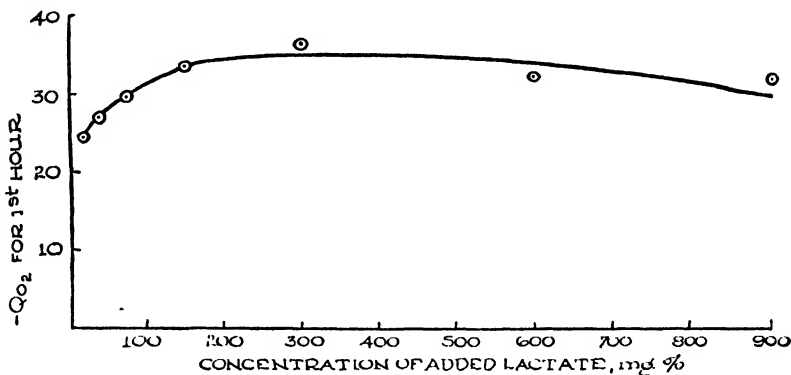
FIG. 1. Respiration during first hour plotted against concentration of lactate in medium. (Note. Points in Figs. 1 and 2 represent averages of 2 flasks; all other points on Figs. represent averages of 3 flasks.)

concentration of added *dl*-lactate. It will be seen that the maximum effect was reached at a concentration of about 300 mg.-% and that higher concentrations of lactate were slightly less effective.

In Fig. 2 are shown the variations of respiratory rate with time in the presence of a number of different concentrations of lactate. It can be seen that there is a tendency for a more rapid falling off of respiration during the first hour in the tissues with added lactic acid than in the control tissues.

It was similarly observed that the addition of 200 mg.-% of glucose to the medium had a maximal effect in increasing respiration. A
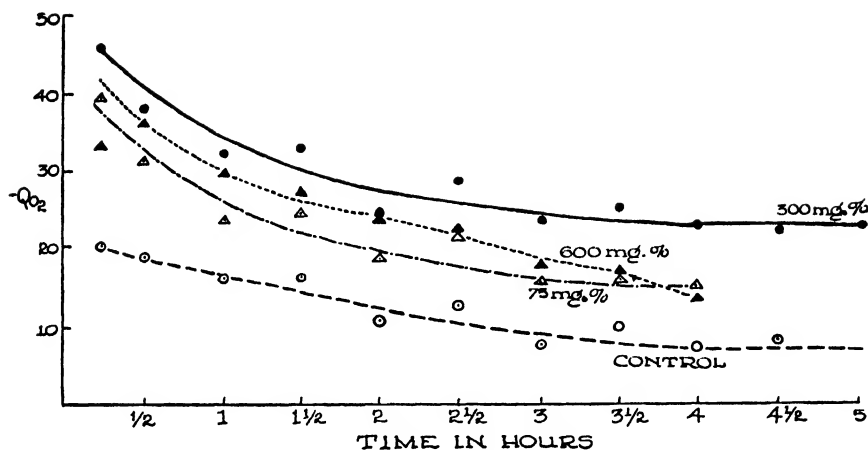
FIG. 2. Respiration of rat kidney slices in oxygen with different concentrations of lactic acid in medium.

comparison of the effect of equal concentrations of glucose and lactate (200 mg.-%) on the respiration is shown in Fig. 3. It can be seen that
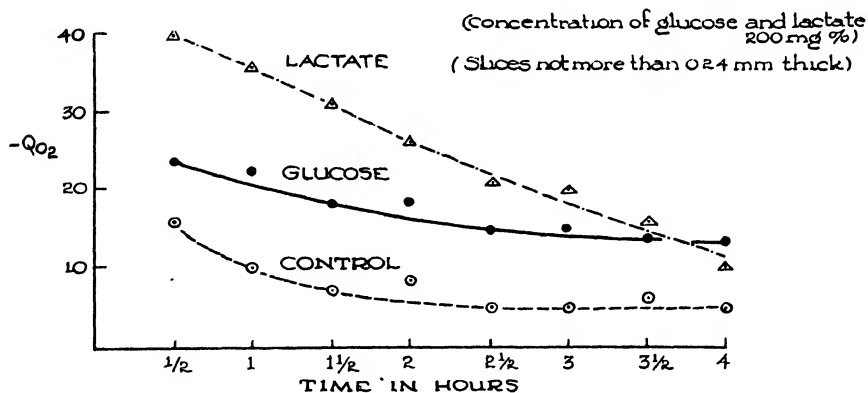
FIG. 3. Respiration of rat kidney slices in oxygen with and without glucose and lactate in the medium.

respiration in the presence of lactate is almost twice that with glucose initially, but with lactate the rate of respiration declines much more rapidly than with glucose, so that at the end of 4 hours it is less than the rate with glucose and approaches that of the control tissues. In this experiment rather thin slices, less than 0.24 mm., were used.

Thicker slices in a medium containing glucose show an almost constant rate of respiration during a 4 hour period. The results of such an experiment are shown in Fig. 4. Thicker slices with lactate do not show
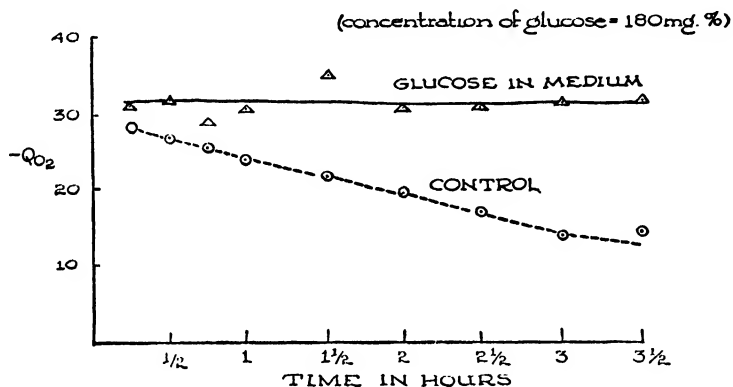
FIG. 4. Respiration of rat kidney slices in air without substrate and with added glucose.

this constancy of respiration rate any more than do thinner slices with lactate.

Figs. 5 and 6 demonstrate, respectively, the effects of adding lactate and glucose to the medium after respiration has proceeded for some time. It will be noted that in each case the rate of respiration was
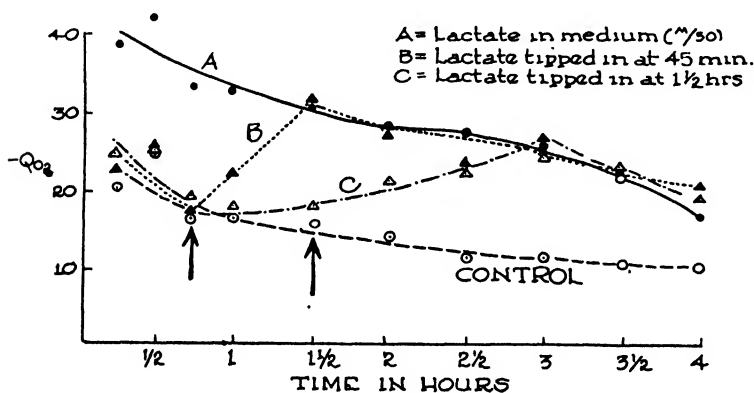
FIG. 5. Respiration of kidney slices in oxygen with lactate in meduim at start and tipped in at 45 and 90 minutes.
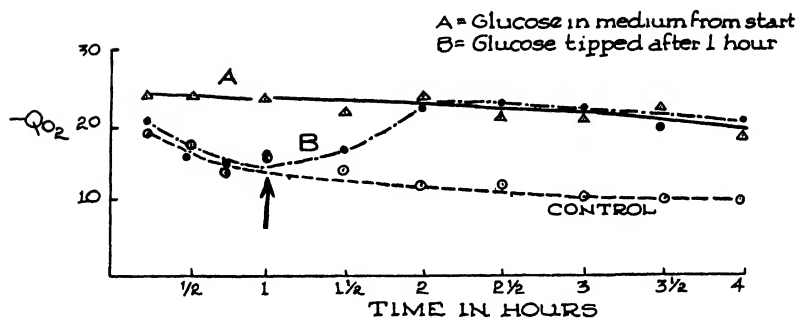
Fɪɢ. 6. Respiration of kidney slices with glucose in medium
and glucose tipped in after 1 hr.

accelerated so as to equal, but not exceed, that of the tissues which had
had access to these substrates from the beginning of the experiment.
Thus, it would seem that the decline in the rate of respiration ob-
served in the presence of added lactate is not due to an appreciable
decrease in the substrate concentration and must result from a decrease
of some other necessary factor or factors.

Discussion

The control curves of the respiration rate previously shown are
fairly characteristic of all the experiments with 100% oxygen and
closely resemble those obtained by Laser (16) with mouse kidney
slices. However, our curves, which illustrate the effects of adding sub-
strate, do not resemble those published by Laser. According to our
experience the rate of respiration in the presence of lactate falls more
sharply than it did in Laser's experiments, although the latter states
that sometimes in his experiments with lactate the rate of respiration
did fall off before 90 minutes. Laser found that, in the absence of
carbon dioxide, the rate of respiration after the addition of lactate did
not equal that of tissues in which lactate had been present initially.
It will be seen in Fig. 5, however, that the rate of respiration after the
addition of lactate equals that of the tissues respiring in the presence
of lactate from the beginning, although in our experiments carbon
dioxide is absorbed by the alkali in the center well.

In our experiments lactate causes a pronounced increase in respira-
tory rate which is poorly sustained. Glucose addition results in a
smaller increase, but the rate is better sustained. These observations

are similar to those published by Gerard (8, 9), which were obtained with *Sarcina lutea*.

Gerard concluded from his experiments that the rapid decline of respiration with lactate was probably due to the exhaustion of some substance necessary for cellular metabolism. Laser (16) also discusses possible reasons for the falling off of respiration and offers the following as possibilities: death of cells, diffusion out of the cell of essential substances involved in respiration, and oxidation of such substances. Some of our experiments seem to indicate that substrate diminution may also be a factor, since, with thicker slices, it was possible to obtain a rather constant rate of respiration for several hours by adding glucose to the medium. Similar observations were made by Fuhrman and Field (7) with liver slices.

With very thin slices, as well as with slices respiring in the presence of added lactate, we always observed an early falling off of respiration. Thin slices may have a high degree of damaged cells, so that cellular death may be an influencing factor. Slices respiring in lactate have such a large initial respiration that some of the substances necessary for respiration may be used up, and this results in a decreased rate of respiration.

II. Effect of Different Oxygen Tensions upon Respiration

The respiration of kidney slices in air and in 100% oxygen, as well as in intermediate oxygen tensions, has been measured with slices respiring with and without added substrate. In all the experiments with substrate added to the medium (a total of 18) there was a difference noted in the respiratory rates at different oxygen tensions. In 14 out of 18 experiments with slices respiring without substrate added this difference was also observed; irregular results were obtained in the other 4. Fig. 7 shows the differences in the respiration rates of tissues respiring without added substrate in air and oxygen and with lactate in air. Fig. 8 shows the results of another experiment in which the tissues respired in a medium containing lactate. Fig. 9 represents the results of a similar experiment with glucose in the medium. In Fig. 10 the relation between respiratory rate and oxygen tension is shown for oxygen tensions intermediate between air and oxygen. These latter curves also illustrate the more rapid falling off of respiration of slices respiring at higher oxygen tensions.
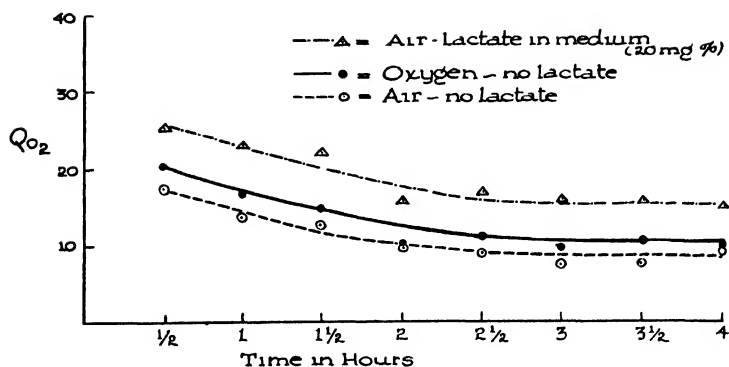
FIG. 7. Respiration of kidney slices in air and oxygen
(slices not thicker than 0.18 mm.).
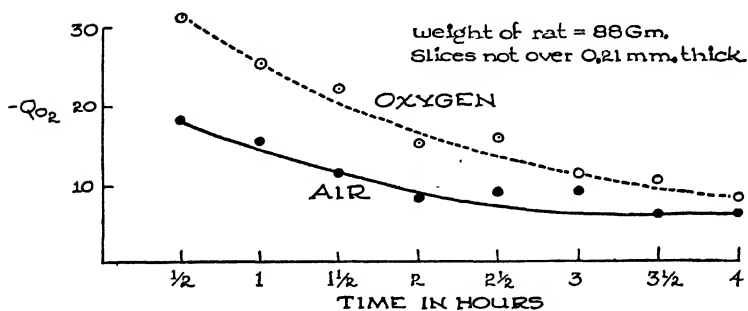
FIG. 8. Respiration of rat kidney slices in air and oxygen with
lactate (200 mg. — %) in medium.
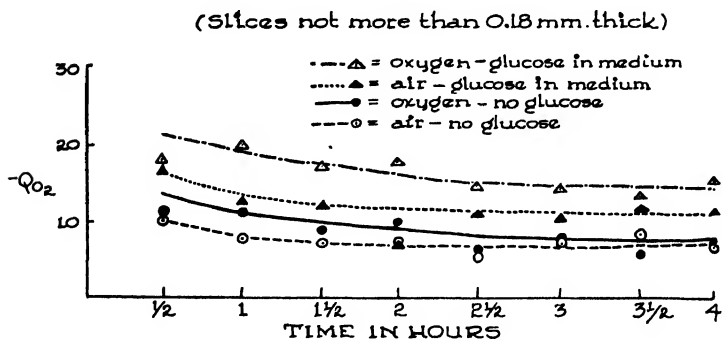
FIG. 9. Respiration of kidney slices in air and oxygen with
and without glucose in medium.

40

30

$-Q_{O_2}$

20

10

30'

60'

90'

120

130

20   40   60   80   100

Per cent oxygen in gas mixture
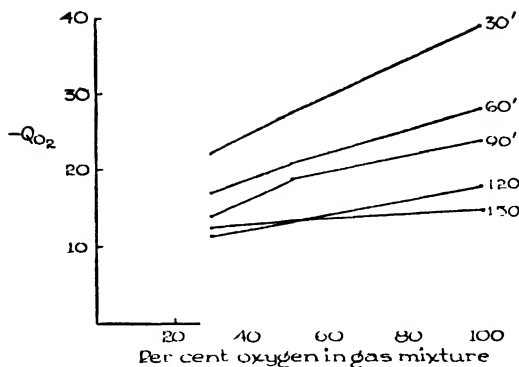
Fig. 10. Respiration rates at different times plotted against oxygen tension.

Discussion

If these results are to be considered significant, the possibility that the diffusion of oxygen is a limiting factor must be excluded. The diffusion of oxygen may be a limiting factor if the speed of shaking is too slow or if the slices are too thick for the oxygen tension used. That the former is not a factor here is indicated by other experiments with the apparatus in which rates of oxygen uptake several times those reported here have been obtained under comparable conditions.

It is not so easy to be certain that the thickness of the slice is not excessive. The formula which Warburg (20) derived for the "limiting thickness" of any tissue slice is:

$$d' = \sqrt{8C_O \times D/A}$$

in which d' is the limiting thickness of the tissue slice, C_O is the oxygen tension in atmospheres, D is the diffusion constant for oxygen through tissues and A is the rate of oxygen consumption expressed as cc./min./cc. of tissue. The limiting thickness thus varies directly with the square root of the oxygen tension and the rate of diffusion, and inversely with the Q_{O_2}. The value of A may be derived from Q_{O_2} by converting the dry weight to the volume of the tissue.

For D, Warburg employed the value obtained by Krogh (15) on the abdominal muscle of the frog. Krogh's constant is based upon a number of assumptions, and it may not be applicable to the diffusion of oxygen through kidney slices. Gerard (8, 9) pointed out that the diffusion constant may differ in different cell zones, and between extracellular and intracellular fluids. Also, Krogh's data were obtained at lower temperatures. Fuhrman and Field (7) and Hill (10) employed the value 1.64×10^{-5} (cc. of O_2/min. through 1 cm.² of tissue with a pressure gradient of 1 atm./cc.) for 37°C., which is the value used in our calculations.

The value of A in Warburg's formula depends upon the rate of respiration of the tissue studied. This is not always appreciated: for example, Laser (16) used the value

obtained by Warburg on liver in estimating the limiting thickness of mouse kidney slices. Yet he reported values for the Q_{O_2} in his experiments as high as 60. Hence, the different respiration rates which he noted with differing oxygen tensions may have been due to excessive thickness of the slices.

The value of A may be calculated from a given Q_{O_2} value using the following formula:

$$A = \frac{Q_{O_2} \times 1000}{1000 \times 60 \times 4.7} = \frac{Q_{O_2}}{282}$$

where 4.7 represents the wet weight/dry weight ratio observed in our experiments. The limiting thicknesses of slices calculated in this manner from Warburg's formula for different rates of respiration and different oxygen tensions are given in Table I.

TABLE I

Limiting Thickness of Kidney Slices Permissible with Different Oxygen Tensions and Different Respiration Rates

(Expressed in mm.)

Q_{O_2}	Oxygen concentration			
	21%	30%	50%	100%
40	.14	.17	.22	.31
35	.15	.18	.23	.33
30	.17	.20	.25	.36
25	.18	.21	.28	.39
20	.20	.24	.31	.44
15	.23	.28	.36	.51
10	.28	.34	.44	.62

We have tried to fulfill the criteria of Warburg by using slices which did not exceed the limiting thickness prescribed by the formula in the preceding paragraph. This has not always been possible. In some experiments (particularly with lactate added to the medium) the initial respiratory rate may be so great that the diffusion of oxygen cannot be excluded as a limiting factor in the respiration of the tissues. At later periods in the experiment, the respiratory rates are slower and the limiting thickness of the tissues is no longer exceeded according to the formula. That the diffusion of oxygen could be a limiting factor at the later periods of the experiment seems unlikely on the basis of the following consideration of Fig. 8. The limiting thickness is at all times well under that required for respiration in 100% oxygen. It can be seen that the rate of respiration in oxygen at the end of 2.5 hours is less than that in air during the first hour. Since the tissues respiring in air initially took up oxygen at a faster rate than that observed later, even in 100% oxygen, the diffusion of oxygen does not seem to be a limiting factor in these later periods, when there is still a difference in the respiratory rates in air and in oxygen.

It has been mentioned that the Q_{O_2} values in our experiments are based upon final dry weight measurements and are higher than they would have been if initial dry weights had been used. Thus, the limiting thickness values as calculated by the formula would be smaller than those based on initial dry weight measurements.

It has also been mentioned previously that in 4 of our experiments, employing very thin slices without added substrate, the respiration was not greater in 100% oxygen than in air. This may have been due to the diffusion of substrate out of the thin slices during their preparation, so that the availability of substrate was the limiting factor. That substrate can be a limiting factor in respiration is shown by the fact that respiration in air in the presence of substrate is greater than that in oxygen without added substrate.

The conclusion that respiratory rate is influenced by oxygen tension is at variance with that of some other workers. Warburg (20) as a result of his earlier experiments on unicellular organisms concluded that respiration is always independent of oxygen tension, provided that diffusion of oxygen is not a limiting factor. His formula for the limiting thickness of slices is based upon this assumption. There is some contrary evidence even with unicellular organisms (21, 8, 11, 12, 23). The investigation of the influence of oxygen tension on the respiration of tissue slices has been difficult because of the problem of slicing tissues thin enough for respiration in low oxygen tensions. Minami (18) investigated the effect of diminished oxygen tension on the respiration of liver slices and found the respiration rate in 100% oxygen to be no greater than that in 50% oxygen. The thinnest slices employed by Minami were 0.23 mm. Laser (17) found in mouse liver, but not in rat retina, rat chorion or mouse sarcoma, a difference in respiration rate with different oxygen tensions. Laser (16) also found differences in the respiration of mouse kindey in 21% and 100% oxygen at the beginning of a given experiment, but not in the later periods. As mentioned previously, it is possible that he exceeded the limiting thickness permissible according to Warburg's formula. More recently, Warren (22) has shown that oxygen tension influences the respiration of bone marrow, and Craig and Beecher (5) have shown a similar influence on the respiration of cat brain slices.

Although it is incumbent on those studying the influence of oxygen tension upon the respiration of tissue slices to fulfill the criteria of limiting thickness as defined by Warburg's formula, it must be remembered that this formula is itself based upon the assumption that respiration is not influenced by the oxygen tension. The oxygen tension at the center of the slice will in low tensions be less than that in the surface cells; thus, the rate of respiration at the center of the slice may fall off before the oxygen tension there is zero.

Some isolated enzymes have been shown to be influenced by oxygen tension. Kohn (13) found this to be the case with tyramine oxidase of pig's liver and Kempner (12) found this true also of d-amino oxidases of kidney. That the oxidation of carbohydrate is also thus affected

seems to be indicated by the differences in respiration observed when lactate and glucose are added to the medium of the respiring tissues.

SUMMARY

1. The respiration of rat kidney slices has been investigated with and without the addition of glucose and lactate under different oxygen tensions.

2. The initial rate of respiration is greater with lactate and (to a lesser extent) with glucose than when no substrate is added. On the other hand, the respiration rate falls more rapidly with lactate and less rapidly with glucose than in the control tissues without added substrate. A constant respiratory rate may be obtained for several hours by using relatively thick slices and adding glucose to the medium.

3. The oxygen tension influences the rate of respiration, which is less in air than in oxygen. Intermediate oxygen tensions are associated with intermediate rates of respiration. The effect is apparent both with and without added substrate (glucose and lactate). The respiratory rate tends to fall off more rapidly in 100% oxygen than in air.

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The Induced Reaction Between Methyl Linoleate and Bixin During Oxidation by Lipoxidase

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INTRODUCTION

Recently we (3) have introduced a method for the estimation of lipoxidase based on the induced, or coupled, oxidation of bixin and soy bean fatty acids. It seemed desirable to investigate this coupled reaction in relation to the reactions formulated by Farmer *et al.* (5, 6, 7, 8) and Bolland *et al.* (4) as to the mode of attack in the autooxidation of methylenic interrupted type of unsaturation such as is present in methyl linoleate.

They have suggested that these reactions involve a displacement or severance of the activated α-methylenic C—H bond resulting in the formation of an olefinic free radical. The displacement presumably requires the aid of molecular oxygen. Under these conditions, resonance between the two 3-carbon forms, —CH=CH—CH— and —CH—CH=CH—, would be possible. The resulting electronic displacement would make possible the appearance of two conjugated isomeric forms depending on whether the double bond remains at the original position or appears at the adjacent C—C bond. This displacement is immediately followed by the incorporation of a molecule of oxygen and an atom of hydrogen with the formation of two isomeric hydroperoxides.

The decomposition of the hydroperoxide is apparently of complex nature and at present imperfectly understood. It appears, however, that the hydroperoxide reacts with the double bonds present to give a hydroxyl group at the former and an epoxy group at the latter. These reactions may be represented as follows:
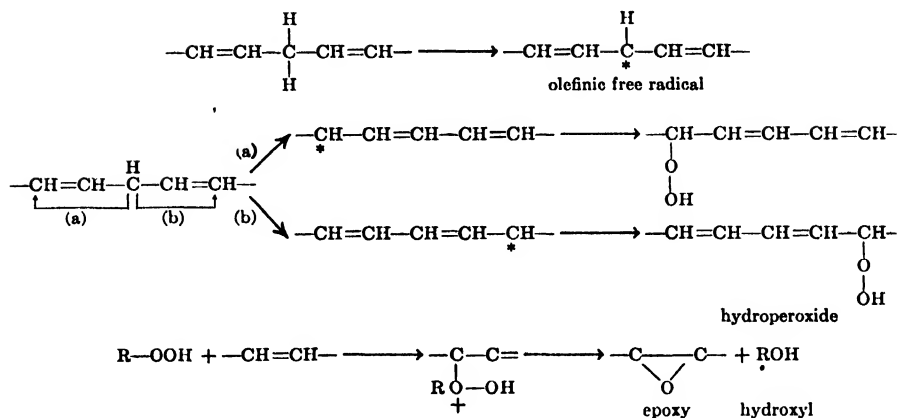
The appearance of these conjugated systems in methyl linoleate can be followed by the changes in the absorption spectrum in the region of 232 mμ as long as the double bonds are not destroyed by the formation of the epoxy groups.

Recently Holman and Burr (9, 10) and Bergstrom (1, 2) have indicated similar electronic displacement of double bonds in methyl linoleate during oxidation by lipoxidase. On the basis of these observations, a series of investigations were undertaken to study the changes taking place in the spectral curves of methyl linoleate and bixin during oxidation with lipoxidase.

EXPERIMENTAL

Procedure

One ml. of stock bixin solution, 0.1 ml. of methyl linoleate-ethyl alcohol and 3 ml. of 0.05 M phosphate buffer, pH 6.5, were mixed in 15 ml. graduated centrifuge tubes. The tubes were placed in a bath at 25°C. and allowed to reach constant temperature. Then 0.15 ml. of lipoxidase solution was added and the digest gently mixed. At various time intervals, 10 ml. of methanol were added, followed by the addition of 0.1 ml. of 20% basic lead acetate. This procedure makes possible the precipitation of the protein present while leaving the methyl linoleate and bixin in solution. The volume of each tube was then adjusted to 15 ml. by the addition of methanol. The tubes were cooled in ice water and centrifuged for 5 minutes at 3,500 r.p.m. to remove the protein precipitate. The clear solutions were then used for analysis with the Beckman spectrophotometer.

The methanol and lead acetate must be added in the order given, as the reverse order will cause the bixin to be adsorbed on the precipitated protein and thus be removed from the solution.

Reagents

Methyl Linoleate. The methyl linoleate used in these investigations was prepared by the method of Rollett (11) from soy bean fatty acids prepared in this laboratory. Saponification and bromination were carried out at low temperatures to minimize possibility of the formation of conjugated systems during the preparation. All distillations were performed in an atmosphere of nitrogen to prevent the oxidation of methyl linoleate.

One g. of the freshly prepared ester was dissolved in 10 ml. of freshly distilled 95% ethyl alcohol and stored under nitrogen in brown bottles at $-20°C$. until needed for the experiments.

Bixin Solution. The bixin solution was prepared by dissolving crystalline bixin (15) in 95% ethyl alcohol so that the final concentration was 0.065 mg./ml. of the solution. A few drops of 0.1 N sodium hydroxide were added to aid in dissolving the bixin.

Purified Lipoxidase. One hundred g. of defatted soy bean meal was extracted with 500 ml. of ice cold distilled water containing 20 ml. of 2 N acetic acid. The material was immediately filtered into graduate cylinders containing 0.5 M Na_2HPO_4, using about 50 ml. of the phosphate for each 250 ml. of the filtrate. Neutral saturated ammonium sulfate solution was then added to the resulting filtrate until a 35% saturated solution was obtained. The precipitate was collected by centrifuging and discarded. Additional ammonium sulfate was added to give a 70% saturated solution of ammonium sulfate. The precipitate, which contains the lipoxidase, was collected by centrifuging and dissolved in 500 ml. of distilled water. The solution was dialyzed against running distilled water in the cold room until free of ammonium sulfate. A 10% solution of basic lead acetate was added until a final concentration of 2% lead acetate was obtained. The precipitate was collected and discarded. The supernatant was again dialyzed to remove traces of lead acetate that might be present. This preparation will generally have been purified from 100- to 110-fold.

Phosphate Buffer. The 0.05 M phosphate buffer of pH 6.5 was prepared by mixing 60 ml. of 0.05 M KH_2PO_4 and 40 ml. of 0.05 M Na_2HPO_4.

Methanol. The methanol was prepared by distillation from KOH and zinc.

RESULTS AND DISCUSSION

Previous investigations on the changes in the spectrum of bixin during oxidation had shown that initially a spectral curve of a dilute solution of bixin has negligible absorption in the ultraviolet regions from 220 mμ to 260 mμ. There are, however, two maxima in the visible region at 455 mμ and 480 mμ. As oxidation proceeds there is a gradual disappearance of the maxima in the visible region, without other peaks appearing in either the ultraviolet or visible regions. This decrease in absorption in the visible region has been shown to be directly related to the disappearance of color in the bixin solution, thus it can be used as a direct measurement of the amount of decolorization that has taken place during the oxidation.

In the oxidation of methyl linoleate, it has been shown that maxima at 232 mμ and 268 mμ appear. The absorption at 232 mμ and 268 mμ increases as oxidation proceeds. Farmer *et al.* (5, 6, 7, 8) have presented evidence to show that the appearance of a maximum at 232 mμ can be associated with the formation of conjugated systems in methyl linoleate. This conjugation is the initial indication of oxidation. The maximum at 268 mμ could not be used in these investigations, as the absorption due to methyl linoleate is not sufficient to be distinguished from the absorption due to bixin in this part of the spectra.

The spectral changes in the bixin solutions and the methyl linoleate were investigated simultaneously by observing the changes in the three regions 232 mμ, 455 mμ, and 480 mμ. Typical changes involved are shown in Fig. 1. The determinations, made at 15 second intervals,
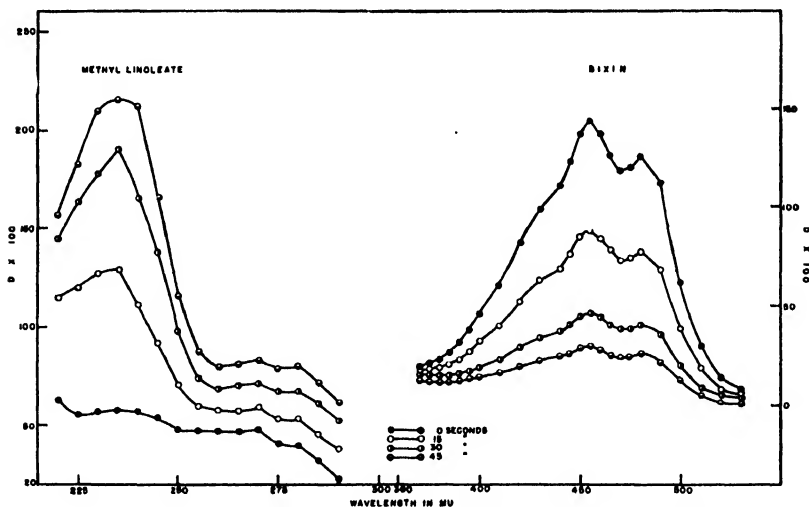
FIG. 1. Changes in the absorption spectra of methyl linoleate and bixin during oxidation by lipoxidase.

show that the maximum at 232 mμ has increased from 58 to 129, 184, and 215, while the maxima at 455 mμ and 480 mμ have decreased from 143 to 88, 46, and 29, and from 125 to 77, 40, and 25, respectively. These results indicate that a relationship probably exists between the initial appearance of the conjugated system of double bonds and the decolorization of bixin as measured by spectral changes in the regions 455 mμ and 480 mμ. This relationship will exist as long as the hydro-

TABLE I

Sample	Time	D readings		Changes in D with time		Ratio 232 mμ 455 mμ
		232 mμ	455 mμ	232 mμ	455 mμ	
I	0 sec.	0.62	2.20	—	—	—
II	5 sec.	0.74	2.05	0.12	0.15	1.2
III	10 sec.	1.05	1.72	0.43	0.48	1.1
IV	15 sec.	1.28	1.54	0.66	0.66	1.0
V	20 sec.	1.57	1.32	0.95	0.88	0.9
VI	25 sec.	1.75	1.23	1.33	0.97	0.8

peroxide is being used for the oxidation of the bixin and not for the formation of epoxy groups in the linoleate. Under the conditions used, decay of the hydroperoxide generally starts after 30 seconds. Since the bixin is more easily oxidized by the hydroperoxide than are the double bonds, the epoxy groups will only appear after the majority of the bixin has been destroyed.
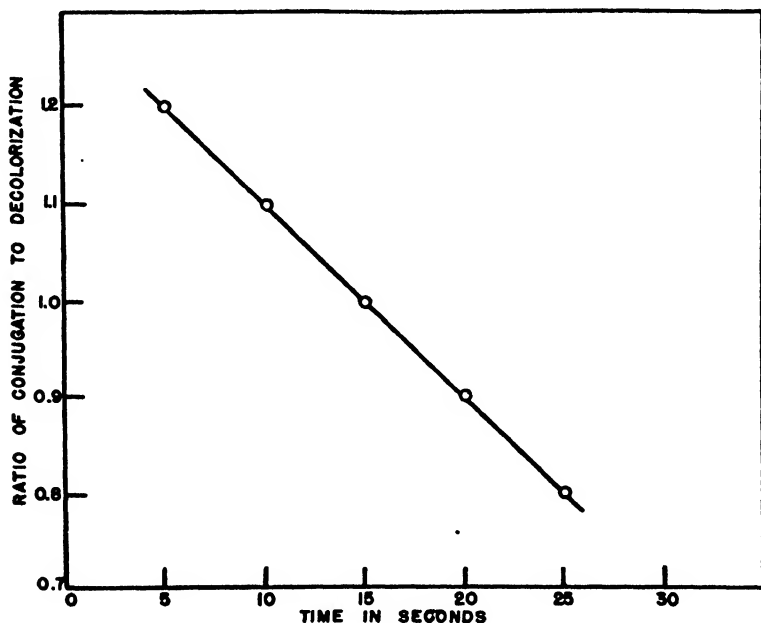
FIG. 2. Changes in the ratio of conjugation to decolorization during oxidation with lipoxidase.

If a direct relationship exists in this initial stage of oxidation, the ratios of the increased absorption at 232 mμ and the decreased absorption at 455 mμ or 480 mμ should be a linear function when plotted against time. Table I indicates typical data obtained from such investigations using concentrated bixin solutions. The ratios obtained by dividing the decrease in D readings at 455 mμ by the increase in D readings at 232 mμ, show a progressional decrease with time (see Fig. 2). Since a linear function is obtained, there is a proportionality between the initial changes in the absorption spectrum of methyl linoleate at 232 mμ and the changes in the absorption spectrum of bixin at either 455 or 480 mμ. Thus, an estimation of lipoxidase activity can be made by observing visibly the rate of decolorization of the bixin solution, measuring the decrease in absorption at 455 mμ or 480 mμ and by measuring the increased absorption of the methyl linoleate at 232 mμ. All three methods have been used in this laboratory for the estimation of lipoxidase activity.

SUMMARY

Evidence has been presented to show that a relationship exists between the spectral changes in methyl linoleate and bixin during the coupled oxidation by lipoxidase. A proportionality exists between the increased absorption due to methyl linoleate oxidation and the decreased absorption due to bixin oxidation and decolorization. An induced or coupled oxidation reaction thus exists between methyl linoleate and bixin during lipoxidase oxidation.

Three methods of estimating lipoxidase activity have been suggested based on the spectral changes of this system during oxidation.

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Synthesis of α-Acetolactic Acid[1]

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INTRODUCTION

During the biochemical conversion of pyruvic acid to acetylmethylcarbinol by some microorganisms and mammalian tissues there is evidence that acetaldehyde and pyruvic acid condense to form an intermediary compound. An enzymatic aldol condensation of pyruvic acid with acetaldehyde would produce α-acetolactic acid, which, upon decarboxylation, would yield acetylmethylcarbinol.

$$CH_3 \cdot CO \cdot COOH + CH_3CHO \longrightarrow CH_3 \cdot C \cdot OH(CO \cdot CH_3) \cdot COOH$$
$$\downarrow$$
$$CH_3 \cdot CHOH \cdot CO \cdot CH_3 + CO_2$$
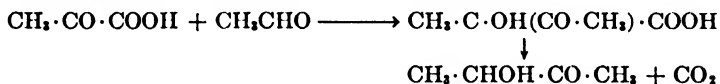
A synthesis of α-acetolactic acid was carried out to test this hypothesis. In this synthesis, oxidation of the labile hydrogen of methyl-substituted ethyl acetoacetate was accomplished with lead tetraacetate, with subsequent hydrolysis of the acetoxy ester.

$$2CH_3 \cdot CO \cdot C(CH_3)H \cdot COOEt + Pb(OAc)_4 \rightarrow$$
$$2CH_3 \cdot CO \cdot C(CH_3)(OAc)COOEt + Pb(OAc)_2$$

$$CH_3 \cdot CO \cdot C(CH_3)(OAc)COOEt + 2NaOH \rightarrow$$
$$CH_3 \cdot CO \cdot C(CH_3)(OH)COONa + CH_3COONa + C_2H_5OH$$
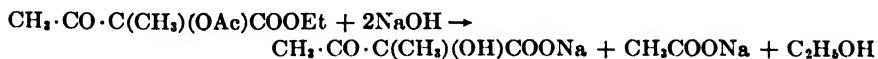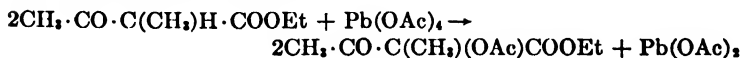
Dimroth and Schweizer (1) have prepared ethyl α-acetoxyacetoacetate by oxidation of one of the labile hydrogens of ethyl acetoacetate with $Pb(OAc)_4$. Weil-Malherbe (2) has studied the hydrolysis of the former ester in detail demonstrating that on acid hydrolysis there is an evolution of CO_2, and on alkaline hydrolysis in air there is

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an absorption of O_2 with production of CO_2. If the hydrolysis is carried out in dilute NaOH under anaerobic conditions and the excess alkali is neutralized before admission of O_2, there is no appreciable absorption of O_2 or evolution of CO_2. Hydrolysis is complete at 25°C. after 1 hour when 2 mols of NaOH have been used. Sodium α-hydroxyacetoacetate, acetic acid, and ethyl alcohol are the products of hydrolysis. Anaerobically, in the presence of 0.1 N NaHCO$_3$, only the acetyl group of the ethyl α-acetoxyacetoacetate was hydrolyzed to yield the α-hydroxy-β-ketonic ester. Under aerobic conditions, however, 2 mols of acetic acid and 1 mol of ethyl hydrogen oxalate were formed. It was postulated that an enediol-ketohydroxy tautomerism occurred with the α-hydroxy-β-ketonic ester and that the enediol ester was autoxidizable with the formation of hydrogen peroxide, which cleaved the ester to 1 mol of acetic acid and ethyl hydrogen oxalate. In contrast to the ester, the unesterified α-hydroxyacetoacetic acid was oxidized very slowly in NaHCO$_3$.

Experimental

The methyl-substituted acetoacetic ester was prepared in the conventional manner (3). Pb(OAc)$_4$ was prepared according to Dimroth and Schweizer (1).

With vigorous mechanical stirring under anhydrous conditions 260 g. Pb(OAc)$_4$ was added in small portions to a mixture of 280 g. thiophene-free anhydrous benzene and 86.4 (0.6 mol) methyl-substituted ethylacetoacetate. During the addition of the Pb(OAc)$_4$ the temperature was not allowed to rise above 35°C., after which the mixture was heated to 40°C. for 5 hours with vigorous stirring. The mixture was allowed to stand an additional 24 hours at room temperature, filtered, and the precipitate washed 5 times with 100 ml. portions of benzene. The washings were combined with the original solution. To remove any acetic acid present in the benzene, it was washed with 100 ml. portions of water until the water was neutral to bromothymol blue. The benzene later was dried over MgSO$_4$, filtered, and the benzene distilled off under reduced pressure. The remaining ester was distilled at a pressure of 1–10 mm. A fraction was obtained at 38°–43°C. which contained mostly unreacted methyl-substituted ester. Another fraction (48 g.) was obtained at 79°–83°C. A fraction remained which appeared to be a highly polymerized yellow oil.

Characterization of the methylacetoxy-substituted ester was performed on the 79°–83°C. fraction which proved to be quantitatively pure. Hydrolysis characteristics were established. NaHCO$_3$ (0.1 M) is sufficiently alkaline to hydrolyze only the acetyl as determined by the liberation of CO_2 from the bicarbonate ion by the acetic acid produced. Similar results were obtained with ethyl α-acetoxyacetoacetate by Weil-Malherbe (2). These experiments were conducted in Warburg respirometer vessels. Each vessel contained 0.2 mM NaHCO$_3$ and 0.1 mM methylacetoxy-substituted ethyl acetoacetate. The bicarbonate ion was determined before and after

hydrolysis. 2240 μl. CO_2 is equivalent to 0.1 mM ester, providing one acid equivalent is produced. In a series of 6 experiments an average of 2252 μl. of bicarbonate ion were consumed in 40 mins. At the end of this period the hydrolytic activity ceased. If the ester linkage were hydrolyzed, a second equivalent would have been formed. In the presence of dilute NaOH 2 acid equivalents are produced (Fig. 1).
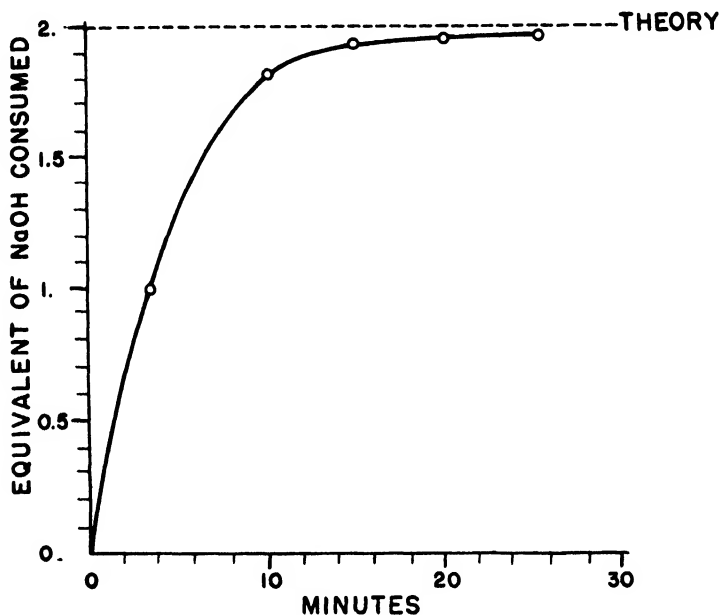
FIG. 1. Hydrolysis of methylacetoxyethylacetoacetate with NaOH. 2.5 mM ester + 5 mM NaOH. Volume 25 ml. Samples titrated at 0, 5, 10, 15, 20, and 25 minutes with 0.009 N HCl.

There is no autoxidation, either of the ethyl α-acetolactate or the free acid. This is to be expected, since the compound cannot tautomerize to the enediol form as can ethyl α-hydroxyacetoacetate.

Solutions, which have been hydrolyzed with 2 equivalents of NaOH, yield CO_2 with aniline (4) or by acidification and boiling as do other β-ketonic acids (5) (Table I).

After the decarboxylation had been accomplished by the acid-heat method (Table I) the solution was neutralized and 14 volumes of steam distillate were collected. The acetylmethylcarbinol was thereby obtained. A periodate oxidation of this distillate was carried out according to the method of Brockman and Werkman (6). Sodium bisulfite was used to trap the acetaldehyde obtained from the oxidation. 0.792 mM of acetaldehyde was obtained. Destruction of the excess periodic acid with thiosulfate, and steam distillation under acid conditions, gave 0.788 mM acetic acid. The theoretical values are 0.8 mM, since acetylmethylcarbinol is oxidized by periodic acid to 1 mol of acetic acid and 1 mol of acetaldehyde.

TABLE I

Determination of α-Acetolactic Acid

Method	Amount of α-aceto-lactic acid	CO_2 obtained	CO_2 theory
Aniline	.05 mM	1114 μl.	1120 μl.
Acid-heat	0.8 mM	34.8 mg.	35.2 mg.

The aniline experiments were performed in Warburg respirometer vessels by the method of Edson (4). Vessels contained 0.05 mM α-acetolactic acid obtained by hydrolyzing the ester with two equivalents of NaOH.

The acid-heat experiments were performed by placing 0.8 mM of the above α-acetolactic acid solution in a reflux apparatus fitted with a drying tube, and collecting the CO_2 in ascarite tubes.

Because of the lability of the free acid to decarboxylation under a variety of conditions, molecular weight determinations were made on the semicarbazone derivative of the acetoxy ester.

To a mixture of 2 ml. of the ester and 2 ml. of water with sufficient alcohol to just dissolve the ester, 1 g. of semicarbazide and 1.5 g. of sodium acetate were added. The mixture was shaken until clear, placed in a boiling water bath which was no longer heated, and allowed to come to room temperature. The tube was then cooled in an ice bath, where crystallization took place. The product was recrystallized 3 times from alcohol and water and dried in a vacuum dessicator over P_2O_5. M.P. 135°C. Molecular weight determinations on the semicarbazone were made by the method of Veibel (7). From the quantity of ammonia produced by the potassium iodate oxidation the molecular weight was determined. The average result obtained from 3 determinations whose deviation was less than 0.1% was: 10.052 mg. nitrogen/185.4 mg. of semicarbazone or 5.42%; theory 5.43%. Molecular weight calculation for the derivative is 259.56 as compared to 259.07 for theory.

Carbon-hydrogen determinations were performed on the semicarbazone: calculated: C, 46.3%; H, 6.56%; found: C, 46.5%; H, 6.61%.

Preliminary results with an enzyme preparation obtained from *Staph. aureus* show that there is a rapid enzymatic decarboxylation of the free acid to acetyl methylcarbinol (8).

SUMMARY

α-Acetolactic acid has been proposed as an intermediate in the biochemical conversion of pyruvic acid to acetylmethylcarbinol.

Synthesis of α-acetolactic acid was accomplished by oxidation of the labile hydrogen in methyl-substituted ethyl acetoacetate.

Chemical characteristics of the compound were established.

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An Enzymatic Assay for Studying the Nutrition of *Drosophila melanogaster*

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The usefulness of the fruit fly, *Drosophila melanogaster,* as a tool for for the study of genetics would be considerably increased if it were possible to culture it on a completely synthetic medium. The more recent developments in genetics, especially those concerned with *Neurospora,* make it clear that complete control of the food substrate is necessary for the proper study of the physiological and biochemical aspects of gene action.

Guyenot (4) and Baumberger (1) showed that the yeast ordinarily present in the laboratory medium used for culturing the fly provides adequate nutrients for the complete development of *melanogaster.* Subsequent work by Wagner (14) made it apparent that the fly is also dependent on yeasts and other microorganisms in nature. Studies made to determine what chemically defined substances present in yeast are necessary for *melanogaster* showed that its nutritional requirements, as far as known, correspond roughly to those of other insects. It requires several members of the B complex (10, 11) as well as cholesterol or ergosterol (13), in addition to a source of protein and other undefined substances. The fat-soluble vitamins are apparently not necessary (6). Schultz *et al.* (9) report having obtained growth of *melanogaster* under sterile conditions on a medium consisting of sucrose, salts, cholesterol, ribosenucleic acid, casein hydrolyzate and the B vitamins.

Investigations on the nutritional requirements of *melanogaster* have been seriously handicapped by the lack of an adequate method for measurement of growth. The methods previously used include measurement of the time required for larvae to pupate, the number of adults developing from a given number of larvae and the length of the larvae after a specified growth period. None of these methods provide

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more than a qualitative test for the adequacy of a culture medium. It was, therefore, the first objective of the present investigation to develop a reasonably rapid and accurate means of measuring the growth of the organism. The method to be described was based on the assumption that a proportionality would exist between the growth attained by a larva and its content of the enzyme adenosine deaminase. This enzyme, which catalyzes the conversion of adenosine to inosine, can be readily determined by the spectrophotometric method described by Kalckar (5) and Mitchell and McElroy (7, 8). Larval growth was used as an index of the adequacy of the test medium primarily because it would give results in the shortest possible time after the laying of the egg. There exists, of course, the possibility that the medium may contain the necessary substances for larval development, but may be deficient in those substances required for the normal development of the pupae.

Experimental

Preparation of Flies and Eggs

The adults of the Oregon R wild type strain used as egg layers were grown in mass culture on a medium containing Fleischmann's dried yeast suspended in 2% agar. Besides the dead yeast, the culture food contained actively growing live yeast and bacteria which were carried along by the flies. Two to three hundred adult flies at least 5 days old were transferred from culture bottles, in which there were good growths of 2–3 day old larvae, into bottles with fresh food. Into these bottles there were introduced microscope slides smeared with 10% starch paste containing 0.5% propionic acid. After two hours the slides were removed, and the eggs deposited on them were washed into a petri dish, with a stream of water. Most of the eggs sink to the bottom and stick to the glass making it possible to decant the water and wash the eggs with fresh water to remove adhering particles of food.

The eggs were freed of living microorganisms by transferring them with a platinum loop to a watch glass containing 75% ethyl alcohol. After 45 minutes they were transferred into the sterile tubes containing the test media.

By using the technique described above with about 10 bottles of flies, it was found possible to collect and sterilize, within two hours, more than 2,000 eggs. It is to be noted that a large number of eggs will be laid consistently in two hours only when the adults used for egg laying are taken off a culture food containing old larvae.

In all experiments reported in this paper the inoculum size per tube was 20 eggs, and each tube contained 5 ml. of test medium. A temperature of 25°C. was maintained during the course of all experiments. The ages of the larvae were determined from the time of the laying of the eggs. This was done because it is possible for eggs to hatch within a range of 18–24 hours after laying, even though the eggs have been laid within a two hour period.

The Adenosine Deaminase Content of the Larvae

The micro method of Mitchell and McElroy (7), adapted from Kalckar (5), was utilized to measure the adenosine deaminase of the larvae grown in aseptic culture. The data given by Mitchell and McElroy (8) for the adenosine deaminase of *Aspergillus oryzae* were used as an aid to setting up the procedure outlined below.

The initial experiments were designed to determine whether the larvae contained conveniently measurable amounts of the enzyme, and if so, whether the enzyme content of the larvae increased with age of the growing larvae. The medium used for these experiments consisted of Flieschmann's dried yeast uniformly suspended in 2% agar. Two sets of tubes were inoculated with eggs, one set having 150 mg. of yeast/5 ml. and the other 12.5 mg./5 ml. Previous work by Tatum and Beadle (12) had shown that the former concentration of yeast was well above the optimal, and the latter definitely deficient, for the flies. Larvae were removed from the tubes beginning at 24×2 hours after egg laying and for varying periods thereafter. Those of similar age were put together in groups of 20 for those less than 60 hours old, and in groups of 10 for those over 60 hours old. Each group was then ground in a glass micro grinder along with 1 ml. of $1/15\ M$ phosphate buffer adjusted to pH 6.5. After grinding, the suspensions were diluted and shaken with 5 or 10 ml. of buffer depending on the size of the larvae. Three ml. of each suspension was then pipetted into a test tube containing 3 ml. of an adenosine solution (40 mg./ml.) in phosphate buffer. This mixture was shaken and divided immediately into two 3 ml. portions. One aliquot (A) was placed immediately in a boiling water bath for 5 minutes to destroy the enzyme, and the other (B) into a 35°C. water bath for 90 minutes. At the end of this period B was removed and the enzyme destroyed by boiling. Both tubes were centrifuged and the optical densities of the two solutions determined at a wave length of 265 mμ in a Beckman spectrophotometer. Mitchell and McElroy (7) give a calibration curve for following the enzymatic deamination of adenosine, and, by using this curve, the difference in optical density between A, in which no deamination had taken place, and B in which deamination had proceeded for 90 minutes, was converted into mg. of adenosine deaminated in B. Dilutions of the enzyme were adjusted to give 20–50% deamination of the substrate in 90 minutes.

Fig. 1 presents the data obtained by using this method of measuring the enzyme concentrations of larvae grown on the two concentrations of yeast. It can be seen that there is an increase in the amount of the enzyme with the age of the larvae. This is true for larvae on the complete medium for approximately the first 80 hours after hatching, but after this the enzyme activity declines with increase in age. The same phenomenon was noted with larvae on the deficient medium, but the decline started at 175 hours.

To determine whether there exists a difference in the enzyme activity in female and male larvae of equal age, Oregon R males were crossed with yellow (a sex linked recessive) females and the larval progeny tested as above. The yellow male larvae are easily distinguished from the heterozygous normal female larvae since the males have light brown mouth parts while these appendages are black in the females. The data in Fig. 1 indicate that there is no difference between the sexes for the larvae less than 90 hours old.

Proportionality of Larval Enzyme Concentration to Yeast Concentration

A basal medium was prepared containing the substances listed in Table I. The casein hydrolyzate in the medium was prepared from Labco vitamin-free casein by hydrolysis with HCl. The solution was adjusted to pH 3.0 with KOH and treated with charcoal (Norit A) until almost colorless. The trace element solution was pre-
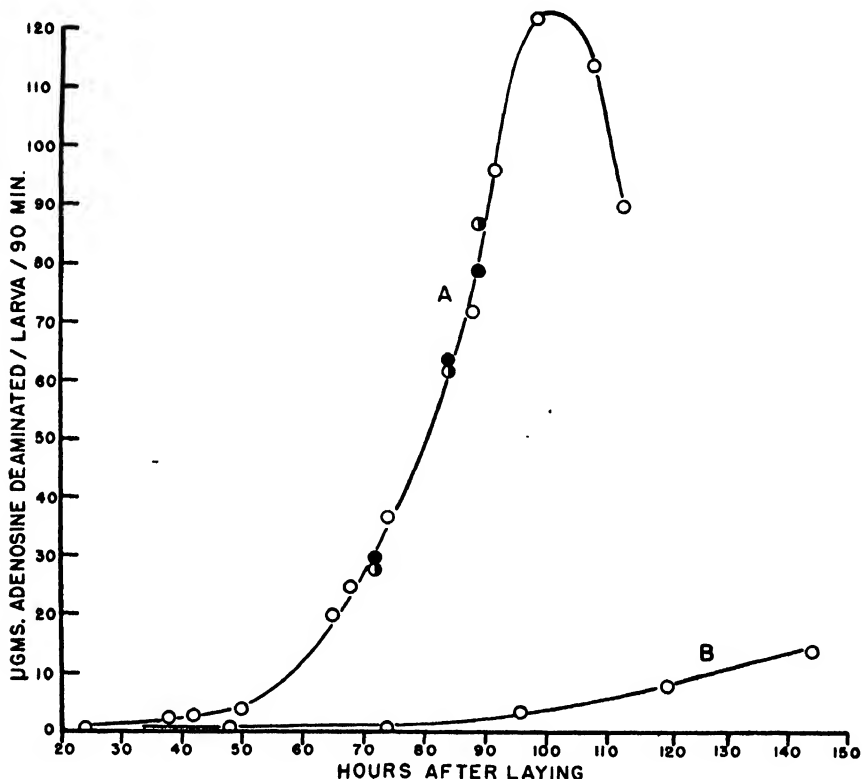
FIG. 1. Adenosine deaminase content of larvae of different ages. Curve A, larvae grown on 150 mg. yeast/5 ml.; curve B, larvae grown on 12.5 mg. yeast/5 ml.

    ○ Normal larvae
    ◑ +/Y larvae (female)
    ● Y larvae (male)

pared and used according to Beadle and Tatum (2). This basal medium is incomplete; larvae do not grow on it and die within 10–12 days.

A series of tubes were prepared containing a substrate made up of the basal medium, various concentrations of Fleischmann's dried yeast and 2% agar. The pH was

TABLE I

Composition of the Basal Medium

Concentrations given in γ/5 ml. unless otherwise specified.

Thiamine................................. 50
Riboflavin................................ 25
Nicotinamide............................. 50
Pyridoxin................................. 50
Ca pantothenate..................... 50
p-Aminobenzoic acid................. 50
Inositol........................ 50
Folic acid 3
Biotin.............. .... 0.25
Choline chloride................... ... .100
Adenine sulfate........ 500
Uracil.......................... 500
Guanine hydrochloride......... 250
Tryptophan............................ 2.5 mg./5 ml.
Cystine............................ .... 0.25 mg./5 ml.
Casein hydrolyzate ....125 mg./5 ml.
Ergosterol...............................250
Sucrose.................................. 25 mg./5 ml.
$MgSO_4$................................ .... 10 mg/5 ml.
$CaCl_2$............................100
$MnSO_4$............................... ..100
KH_2PO_4...... .... 10 mg./5 ml.
NaCl....................................100
Trace elements

adjusted to 5.5 with KOH or HCl. Eggs were introduced and, after 84 hours, the enzyme concentrations of the hatched larvae were determined, using groups of 10. Data from a representative experiment are given in Fig. 2. Repeated experiments have demonstrated an extreme deviation of about 10% from the assumed straight line shown in the data plotted. The value of 55 γ of adenosine/larva/90 minutes is maintained up to yeast concentrations of 300 mg./5 ml.

TABLE II

Pupation Time on Different Concentrations of Yeast

Mg. yeast /5 ml.	Pupation time determined as hours after egg laying							
	10	20	30	40	50	60	70	80
Time of pupation	154–172	130–142	106–118	106–118	106–118	100–112	100–112	100–112

Time of pupation was noted for larvae grown on various concentrations of yeast. These data are presented in Table II, and show (1) that there is a rough correlation between pupation time and the data in Fig. 2, and (2) that use of pupation time as an
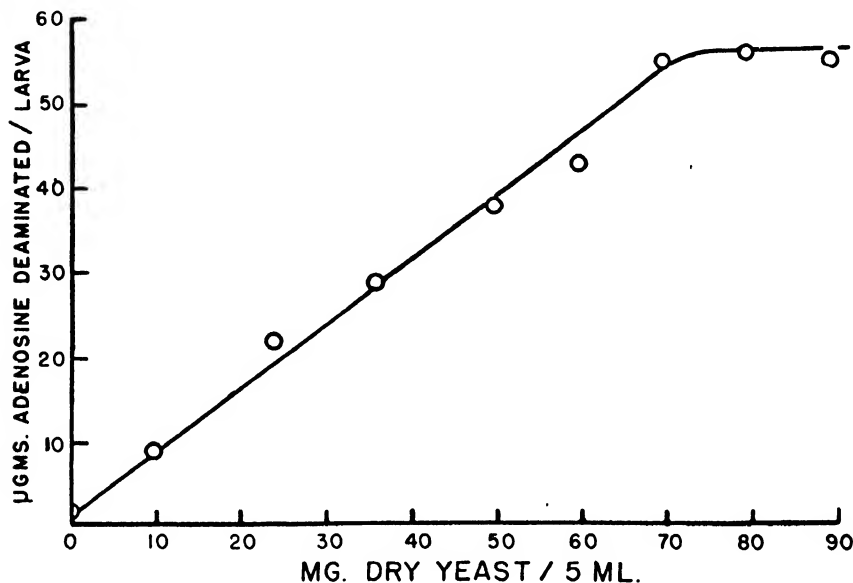
FIG. 2. The relationship between the adenosine deaminase content per larva and the concentration of the yeast in the medium.

assay method is at best only qualitative, besides requiring considerably more time than the enzymatic method.

Investigation of Nutritional Requirements

The basal medium described in Table I includes the known B complex vitamins and, presumably, an adequate supply of amino acid and nucleic acid constituents. However, as previously indicated, this medium does not support significant growth of the larvae. Therefore, extracts of various food sources were investigated as to their adequacy in supporting growth of the larvae when added to basal medium. Some results are reported in Table III. Larval mash and trypsin digests of yeast were investigated originally with *Drosophila hydei* by Chu (3) who reported that tryspin hydrolysis freed a factor which was necessary for larval growth. The data in Table III, however, indicate that enzymatic hydrolysis does not free all of the substances necessary for normal growth of *melanogaster*.

Ribonucleic acid was reported by Schultz *et al.* (9) to be effective in stimulating larval growth when added to a medium similar to the basal medium employed in this work. The results presented in Table III do not support this observation.

TABLE III

Results From Assaying Various Food Sources

Weights given are wet weights unless otherwise specified. Weights given for fractions are equivalent weights of original material from which fractions were derived. Assay value stands for γ adenosine deaminated/larva/90 minutes.

Test fraction + basal medium	Mg./5 ml.	Assay value	Pupation
Basal medium alone	—	2.0	None
Casein hydrolyzate	125	2.2	None
Skimmed milk, dry weight	25	1.8	None
	250	2.5	11 days
Basamin-Busch, dry weight	125	6.5	11 days
Spinach, water extract	2500	2.6	None
Water extract of hog's liver	375	5.2	10 days
	750	9.4	8 days
	1875	11.	8 days
Filtrate from liver autolyzate	650	3.3	11 days
	1300	4.0	10 days
	1800	2.2	10 days
Water extract of dried yeast	100	2.0	12 days
	300	4.5	10 days
2% Ammonia extract of dried yeast	100	4.3	8 days
	300	11.	6 days
	500	12.	6 days
Larval digest of dried yeast	250	7.8	7 days
	1000	7.5	7 days
	2000	6.7	7 days
Trypsin digest of dried yeast	250	13.	6 days
	1000	12.	6 days
Ribonucleic acid (laboratory prep.) dry weight	5	2.9	None
	10	2.4	None
Ribonucleic acid (Eastman)	5	2.2	None
	10	2.4	None

Since yeast provides a good source of nutrients for the larvae of melanogaster, preliminary experiments and fractionations were carried out in an effort to establish the nature of the required substances. Autolysis at 25°C. of fresh baker's yeast cytolyzed with ethyl acetate at 25°C. apparently destroys a necessary factor or factors as is shown by Table IV. However, if the yeast was boiled for 10 minutes immedi-

TABLE IV

Results from Assaying Fresh Baker's Yeast

Weights given as in Table III. Assay value stands for γ adenosine deaminated/larva/90 minutes.

Test fraction	Mg./ 5 ml.	Assay value
Autoclaved fresh yeast	40	14
	50	18
	100	36
	150	48
	200	65
	300	61
Autolyzed 0 hours	50	22
	100	36
Autolyzed 2 hours	50	14
	100	27
Autolyzed 6 hours	50	15
	100	23
Autolyzed 24 hours	50	8.3
	100	18
Water-alcohol extract	100	2.2
	200	2.0
	300	3.0
Residue after extraction, dry weight	50	17
	100	18
	200	16
Combination: Extract	200	
Residue (dry wt.)	50	52

ately after cytolysis, the degradation process was stopped. One pound of fresh yeast was cytolyzed, boiled and then washed successively with water and 95% ethyl alcohol. The alcohol was distilled off from the alcohol extract and the residue added to the water extract. Table IV shows that the combined water-alcohol extract was inadequate alone and that the residue had lost some activity, but almost all activity was regained on combining the two. These results indicate that there are at least two factors, one water-alcohol soluble, and the other either water-alcohol insoluble or bound to an insoluble component.

Work is now in progress on the isolation of the growth factors indicated.

DISCUSSION

An alternative to the enzymatic method described would be the use of the dry weight of the larvae. Dry weights were determined for 84 hour larvae grown on 40 mg. and 70 mg./5 ml. concentrations of dried yeast; 250 larvae washed free of food were used in each case. The results were 0.115 mg. and 0.192 mg. dry weight/larva for 40 mg. and 70 mg. yeast concentrations, respectively. Using this method would require, therefore, at least 100 or more larvae to get significant dry weight determinations with the ordinary analytical balance, for the top half of the assay curve given in Fig. 2. By extrapolation, 2,000 larvae would be needed for assaying the basal medium alone. In addition to the large number of larvae required compared to the usual 10 needed with the enzymatic method, it should be noted that the larvae burrow in the food making it necessary to wash them before determining weights. This tedious step is not necessary when using the enzymatic method, as the food substrate contains no active deaminase even after the larvae have developed in it.

SUMMARY

The adenosine deaminase content of the larvae of *Drosophila melano-gaster* may be determined quantitatively. The enzyme content of the larvae increases with age and a proportionality exists between the larval enzyme concentration at a given age and the yeast concentration of the medium, provided the yeast concentration is below the optimum. These relationships indicate a proportionality between the enzyme content of the larva and the amount of growth attained, and, assuming this, an enzymatic assay has been developed to estimate the effect of various nutrients on the growth of the larvae. Some results of the application of the assay have been presented.

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Folic Acid and the Cholinesterases

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INTRODUCTION

In 1946 Davis (1, 2) claimed that hyperchromic anemia can be produced in dogs by the daily administration of choline or acetylcholine and that this anemia is abolished by the use of liver extract or pteroylglutamic acid. Clarkson and Best (3) have failed to demonstrate that choline, in the amounts prescribed by Davis, causes the production of a macrocytic anemia when added to a stock diet providing approximately 429 mg. of choline daily. They suggest that the explanation for Davis' findings must be sought elsewhere.

Davis showed in his experiments that "an acetylcholine-like activity" is present in the serum of anemic dogs, and that this activity is abolished or diminished after the onset of treatment with antianemic substances. In subsequent papers (4, 5) he presents evidence that both liver extract and pteroylglutamic acid increase the cholinesterase activity of the plasma of dogs and human subjects.

In Davis' experiments a high concentration of acetylcholine was used as substrate in estimating cholinesterase activity and the activity exhibited by the plasma of dog and man towards this substrate is due predominantly to the activity of pseudocholinesterase (6). Selective inhibition of the activity of this enzyme has shown that it is not essential for the removal of acetylcholine *in vivo* (7), symptoms indicative of acetylcholine accumulation appearing only when a significant depression in the activity of true cholinesterase has been attained (7, 8). Therefore, the validity of Davis' hypothesis that the increase in cholinesterase activity brought about by antianemic substances counteracts the increase in circulating acetylcholine will depend on the demonstration that liver extract and folic acid augment the activity of true cholinesterase.

In the present investigation an attempt was made to determine whether the changes in acetylcholine-hydrolyzing ability induced by folic acid *in vitro* and *in vivo*, as reported by Davis, were due to changes in the activity of true cholinesterase or pseudocholinesterase.

Estimation of Cholinesterase Activity

Enzymatic activity was measured manometrically at 37°C. by the Warburg method, in a medium of 0.025 M NaHCO$_3$ saturated with 5% CO$_2$ in N$_2$ (pH 7.4). The activity of true cholinesterase and pseudocholinesterase was measured, as described by Mendel, Mundell and Rudney (6), by the rates of hydrolysis of acetyl-β-methylcholine (Mch) (0.03 M) and benzoylcholine (Bch) (0.006 M), respectively. In all cases controls were run for spontaneous hydrolysis of the substrate employed.

EXPERIMENTAL RESULTS

1. The Effect of Dietary Intake of Folic Acid on the Activity of True Cholinesterase and Pseudocholinesterase in the Plasma of Rats

Experiments were made to ascertain whether the changes in the cholinesterase activity of the plasma observed by Davis after administration of folic acid or following incubation of plasma with folic acid are related to the level of dietary intake of this compound.

Male rats of the Wistar strain (100–130 g.) were divided into two comparable groups and kept in individual cages with a false bottom of coarse wire screen. Group M-0 received the basal diet without any supplement of folic acid. Group M-1 was maintained on the same diet and a daily supplement of 25 γ of folic acid was administered subcutaneously. Weighed amounts of fresh diet were given daily and the following morning the amount left over and the scatter were weighed. From these data the individual daily food consumption was calculated. The rats were group pair fed, the average daily intake being approximately 14 g. The experiment was continued for 10 weeks. At the end of this period the weight curve for the animals in group M-0 had been falling off for 8–10 days.

Basal Diet

	Per cent
Casein (Labco)	20
Celluflour	2
Salt mixture (9)	4
Sucrose	68.8
Corn oil with added vitamin E	3
Choline chloride	0.1
Inositol	0.1
Succinylsulfathiazole	1.0
Vitamin mixture	1.0
Cod liver oil concentrate	0.015
Biotin (20 γ/100 g. diet)	

The vitamin mixture consisted of:

Thiamine HCl.......................................100 mg.
Riboflavin........................... ....100 mg.
Nicotinic acid.....................................500 mg.
Pyridoxine............................... ....100 mg.
Calcium pantothenate................... ....500 mg.
p-Aminobenzoic acid................... ....100 mg.
2-Methyl-1,4-naphthoquinone...... .   .   . 50 mg.

which was made up to 100 g. with very finely powdered sucrose. With a daily intake of 10 g. of diet, a rat would receive 100 γ of thiamine hydrochloride and proportional amounts of the other vitamins. An aqueous solution of crystalline biotin (25 γ/cc.) was sprinkled over the celluflour and air dried before incorporation with the other ingredients. The cod liver oil concentrate (Ayerst, McKenna and Harrison) contained 200,000 i.u. vitamin A and 50,000 i.u. vitamin D/ g. The vitamin E (α-tocopherol acetate, 2 mg./ 10g. diet) was mixed with the corn oil.

Solutions of folic acid[1] were made up thrice weekly, complete dissolution of the compound being obtained by rendering the medium slightly alkaline with a few drops of $N/1$ NaOH and subsequently restoring it to neutrality by the addition of a small amount of sodium acid phosphate.

The experiment was terminated by killing the animals by exsanguination through the jugular vein. A sample of plasma obtained from the oxalated pooled blood of 2 animals was in each case used for the determination of enzymatic activity.

The results of this experiment are given below in Table I.

It will be seen from the results outlined in this table that no significant difference exists between the activity of either the true cholinesterase or the pseudo-cholinesterase in rats on a folic acid-deficient and on a folic acid-supplemented diet.

The level of dietary intake of folic acid in male rats, therefore, has no effect on the activity of either the true cholinesterase or the pseudo-cholinesterase in the plasma of these animals.

2. The Effect of Incubation with Folic Acid on the Activity of the Cholinesterase in the Plasma of Rats

Samples of oxalated plasma obtained from rats in groups M-0 and M-1 maintained on folic acid-deficient and folic acid-supplemented diets as outlined in § 1 were used. The sample was divided into 2 equal parts, one of which was incubated for 2.5 hours at 37°C. with folic acid in a concentration which allowed 1 cc. of plasma to be exposed to the action of 0.15 mg. folic acid, as described by Davis (4); the second

[1] We are indebted to Dr. T. Jukes and Lederle Laboratories for the folic acid (Folvite) used in these experiments.

TABLE I

Activity of the Cholinesterases in the Plasma of Male Rats on Folic Acid-Deficient and Folic Acid-Supplemented Diets

Group	No.	Activity (μl. CO_2 evolved by 1 cc. plasma in 20 min.) towards	
		Mch[a]	Bch[b]
M-0	401 402	35	25
	403 404	46	18
	405 406	49	18
	407 408	43	26
	409 410	60	28
	411 412	38	21
	Average	46	23
M-1	413 414	46	29
	415 416	51	41
	417 418	45	12
	419 420	44	20
	421 422	46	25
	423 424	43	16
	Average	46	24

[a] Mch, acetyl-β-methylcholine.
[b] Bch, benzoylcholine.

part was mixed with a volume of water equal to that in which the folic acid was dissolved, and incubated under similar conditions.

Determinations of the activities of true cholinesterase and pseudo-cholinesterase in the samples treated in this manner revealed that incubation with folic acid (0.15 mg./cc. of plasma) caused no increase in the activity of either enzyme in the plasma of rats maintained on a folic acid-deficient or on a folic acid-supplemented diet.

3. The Effect of Administration of and Incubation with Folic Acid on the Activity of the Cholinesterases in the Plasma of Dogs Maintained at a High and a Low Nutritional Level

To determine whether dietary factors other than folic acid could be responsible for the observation made by Davis that the acetylcholine-hydrolyzing ability of the plasma of anemic dogs is increased after incubation with folic acid, the activity of the cholinesterases in the plasma of 2 groups of dogs which had been maintained on different diets for a relatively long period was determined before and after incubation with folic acid.

Group I consisted of 3 normal dogs maintained for at least three months on the diet used by Davis in his experiments (2).

Group II was made up of 3 normal dogs which received over a three month period the following daily ration:

Ground raw meat[2]	400 g.
Sucrose	80 g.
Bone meal	10 g.

This diet was supplemented twice a week with the following ingredients:

Dried brewers' yeast	10 g.
Cod liver oil[3]	15 g.
Tomato juice	120 g.

Samples of oxalated plasma from dogs of both groups were divided into 2 parts, one part (S_W) being incubated at 37°C. for 2.5 hours with distilled water, the second (S_F) with folic acid in a concentration such that each cc. of plasma was exposed to the action of 0.15 mg. of folic acid, as Davis directed (4). Subsequently, the activities of the cholinesterases were determined, the results obtained being outlined in Table II.

It will be seen in these experiments that the activities of the cho-linesterases in the plasma of dogs maintained on two different dietary

[2] Sold under the trade name of Canada Packers Dog Chum.

[3] The cod liver oil contained 600 i.u. Vitamin A and 85 i.u. Vitamin D/g.

TABLE II

The Effect of Incubation with Folic Acid on the Activities of the
Cholinesterases in the Plasma of Dogs on Varied Diets

Group	Animal	Sample	Activity (μl. CO_2 evolved in 20 min. by 1 cc. plasma) towards	
			Mch	Bch
A	I	W	57	284
		F	58	282
	II	W	—	245
		F	—	247
	III	W	50	310
		F	49	307
B	I	W	68	387
		F	68	391
	II	W	. 49	210
		F	49	211
	III	W	62	218
		F	60	219

regimes are unaffected by an initial incubation with folic acid for 2.5 hours at 37°C.

When folic acid (15 mg.) was administered orally to dogs I and III in Group A, and cholinesterase activities were determined before and 5 hours after this procedure, it was found that the activity of both true cholinesterase and pseudocholinesterase in the plasma remains unchanged.

4. The Effect of the Oral Administration of Folic Acid on the Activity
of the Cholinesterases in the Sera of Human Subjects

Davis and Hamilton (5) report that 5 hours following the administration of a single oral dose (15 mg.) of synthetic folic acid they have observed increases of 14.3–32.3% in the cholinesterase activity of the plasma. Table III shows the levels of true cholinesterase and pseudocholinesterase in the sera of 5 normal males before and after the ingestion of a 15 mg. dose of folic acid. The total activity toward

TABLE III

The Effect of the Ingestion of Folic Acid on the Activity of the Cholinesterases in the Sera of Human Subjects

Subject	Sample no.	Activity (μl. CO_2 evolved by 0.1 cc. serum/20 min.) towards		
		Ach	Bch	Mch[a]
D. F.	I	159	55	19
	II	164	56	22
	III	139	51	19
M. E.	I	186	64	22
	II	168	59	23
	III	143	53	23
M. C.	I	112	38	17
	II	114	38	18
	III	113	38	17
N. T.	I	145	52	20
	II	168	56	21
	III	160	55	21
H. R.	I	166	57	19
	II	156	54	23
	III	152	54	22

[a] Activity of the true cholinesterase was estimated in 1 cc. instead of 0.1 cc.

acetylcholine is also included. Two control samples were removed from the subject (S_I and S_{II}), the second sample being taken 24 hours after the first. Following the removal of Sample II, 15 mg. of synthetic folic acid was ingested and 5 hours later a third blood sample (S_{III}) was taken.

The results outlined in the above table demonstrate that the oral administration of folic acid to normal male subjects fails to produce any changes in the activity of either true cholinesterase or pseudo-cholinesterase in excess of those which can be considered as normally-occurring fluctuations in the levels of these enzymes.

SUMMARY

Incubation of the plasma of rats or dogs with folic acid fails to cause an increase in the activity of either true cholinesterase or pseudo-cholinesterase.

The oral administration of 15 mg. of folic acid produces no significant change in the activity of the cholinesterases in the plasma of dogs or men.

Under the conditions of this experiment no evidence could be obtained in support of the claim that folic acid probably acts by increasing the cholinesterase activity of the body.

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Effect of Phosphate and Other Anions on the Enzymatic Desamidation of Various Amides

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INTRODUCTION

The enzymatic desamidation of glutamine in rat liver digests is relatively slow. Addition of sodium chloride, nitrate, or bicarbonate, results in no appreciable change in rate. When, however, phosphate, arsenate, or sulfate is added to the digest, a considerable acceleration in the desamidation of glutamine occurs (1). That this effect is enzymatic is supported by the absence of any desamidation of glutamine in the presence of heated extracts plus added anions, and by the linear relation of the rate of desamidation to liver extract concentration at constant phosphate concentration (1). No evidence of pyrrolidone carboxylic acid formation is observed, and the ammonia which appears in the digests can be accounted for by a corresponding decrease in amide nitrogen. The pH at maximum activity in the presence of added salt (8.0–8.2) is very close to that in the absence of added salt.

Pyruvate added to a liver digest with glutamine also results in an acceleration of enzymatic desamidation (2–7), but the conditions under which this occurs can be readily separated from those under which phosphate exerts its accelerating effect (6).

In contrast with the accelerating effect of phosphate upon glutamine desamidation, the desamidation of benzoylarginine amide in digests with several rat tissues containing added phosphate is considerably retarded (8, 9). In the presence of 0.066 M phosphate, aqueous extracts of spleen, kidney, liver, brain, pancreas, and muscle, hydrolyze benzoylarginine amide at about one-third the rate in the absence of added phosphate. It was considered of interest to investigate further the effect of various anions upon the enzymatic desamidation of several types of amides in digests with different rat tissues. The results of such studies form the bulk of this report.

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EXPERIMENTAL

The tissues from several hundred male rats (Buffalo stock) were employed in these studies. The animals were sacrificed by decapitation, and the tissues removed and immediately ground in a mortar with clean sand. The homogenized mass was then taken up in distilled water and the extract lightly centrifuged. The supernatant served as the source of the enzymes concerned, and was never allowed to stand for more than one-half hour before use.

The amides employed have been described in earlier papers (2–7, 10), and were made up in aqueous solutions at the concentrations designated. The digests consisted of 1 cc. of extract, plus 2 cc. of buffer at the pH designated and which may or may not contain added anion, plus 1 cc. of either water or substrate solution. The buffer employed at pH ranges from 3 to 8 contained veronal acetate plus 0.1 M sodium chloride (Michaelis). The buffer employed above pH 8 was the Sörenson glycine-NaOH-NaCl. The pH of the digestion mixture was determined before and at the end of the incubation period. Temperature of incubation was 37°C. The salts employed were disodium phosphate, disodium arsenate, disodium sulfate, sodium ethylphosphonic acid, sodium methylarsonic acid, sodium benzenesulfonic acid, sodium pyruvate, and sodium pyruvoylglycinate.[1] These salts were dissolved in the buffer solutions, and the pH adjusted to that value which would yield the desired pH when the buffer mixture was added to the tissue extract. Considerable experience was needed before such adjustments became adequate, for the conditions varied not only with respect to the pH desired but also with respect to the particular tissue used.

The pH for optimum desamidation of glutamine, isoglutamine, and asparagine in liver extracts is about 8.1 (6), that of benzoylarginine amide is about 6.2 (9), and that of glycine amide, alanine amide, and leucine amide is about 9.0 (10).

Enzymatic activity was determined by measuring the ammonia evolved in the digests over that in the tissue extract controls (1, 2). The substrates were entirely stable in the presence of heated tissue extracts whether salts were added or not.

Effect of Anions on Glutamine

The effect of various added salts on the enzymatic desamidation of glutamine in digests with fresh aqueous extracts of different rat tissues is described in Table I.

[1] Sodium pyruvate is most readily prepared by dissolving 50 g. of freshly distilled pyruvic acid in one liter of 95% alcohol followed by the addition, with stirring, of a solution of 16 g. of NaOH in 40 cc. of water. The crystalline product which immediately appears is filtered off, washed thoroughly with alcohol and ether, and dried. The white, glistening product is tested spectrophotometrically for purity and should yield a value for the molar extinction coefficient at 2200 Å of close to 3,000 (cf. 11) (5 × 10⁻⁴ M).

The pyruvoylglycine used was part of the preparation described previously (m.p. 89°C.) (2, 12). It was brought to pH 7.0 in aqueous solution by the addition of dilute NaOH. The spectrophotometric characterization of this compound is described (11).

TABLE I

Effect of Added Anions on the Desamidation of L-Glutamine in Aqueous Rat Tissue Extracts[a]

Added anion	Micromoles $\times$ 10 ammonia N evolved in digests with				
	Liver[b]	Kidney[c]	Spleen[b]	Brain[b]	Skeletal muscle[b]
Buffer alone	20	62	42	22	0
Phosphate	84	62	110	96	0
Arsenate	78	61	98	78	0
Sulfate	74	62	102	74	0
Nitrate	21	61	44	21	0
Ethylphosphonate	38	60	96	68	0
Methylarsonate	19	60	40	22	0
Benzenesulfonate	19	62	38	18	0
Pyruvate[d]	48	60	42	20	0
Pyruvoylglycinate[d]	19	61	42	22	0

[a] Digests consisted of 1 cc. aqueous tissue extract, plus 2 cc. veronal acetate-NaCl buffer with and without added anions, plus 1 cc. of either water or 0.014 M glutamine solution. Final concentration of added anions in the digest 0.01 M. Incubation period 1 hour at 37°C.; pH of each digest carefully set at 8.1 at the start of the digestion period. At the end of the digestion the pH of each digest did not vary by more than $\pm$ 0.02.

[b] Each cc. of extract equivalent to 330 mg. fresh tissue.

[c] Each cc. of extract equivalent to 82 mg. fresh tissue. Prior dialysis of rat kidney extracts before setting up the digests did not result in an increase in desamidation in the presence of added phosphate.

[d] At 0.023 M concentration.

Although kidney extracts contain the most active source of glutaminase (13, 2), no increase in the desamidation of glutamine in the presence of added anions could be observed. Dialysis of kidney extracts against distilled water results in a slight loss in activity in desamidating glutamine, but this activity is still not enhanced when 0.1 M phosphate is added. It is not certain whether extracts of skeletal muscle are completely devoid of glutaminase activity, but, if any activity is present under the conditions used, it is too low to measure with any degree of accuracy.

With extracts of rat liver, spleen, and brain, the desamidation of glutamine is considerably accelerated by the addition of phosphate, arsenate, sulfate, and ethylphosphonate at 0.01 M concentration.

Methylarsonate, benzenesulfonate, and nitrate do not accelerate the enzymatic desamidation of the substrate in these tissues. Of all the tissues studied, added pyruvate produces an increased desamidation of glutamine only with extracts of liver. Pyruvoylglycinate is ineffective in this respect (*cf.* 2).

Relation of pH to Desamidation of Glutamine with Brain Extracts

The pH at optimum activity for the desamidation of glutamine in liver extracts with and without added phosphate was noted to be about 8.1 (1, 6). With extracts of rat brain, a more alkaline optimum, namely,
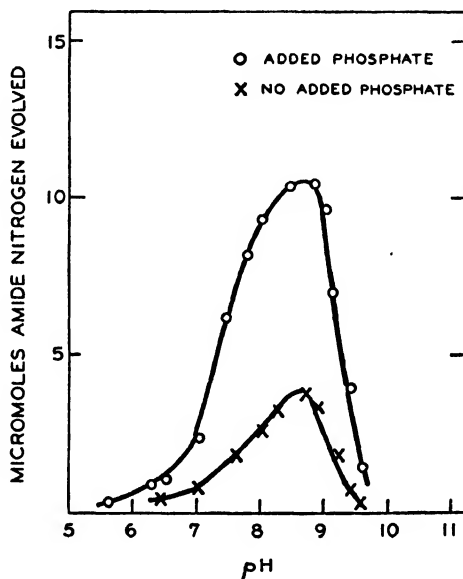
FIG. 1. Relation of pH of the digest to the desamidation of glutamine in rat brain extracts with and without added phosphate. Digests consisted of 1 cc. fresh aqueous rat brain extract, plus 2 cc. veronal acetate-NaCl or glycine-NaOH-NaCl buffer with and without added phosphate, plus 1 cc. of either water or 0.014 M glutamine. Final concentration of added phosphate was 0.01 M. Period of incubation 1 hour at 37°C.

at about pH 8.6 is noted (Fig. 1). This optimum pH is noted whether or not added phosphate is present.[2]

[2] Krebs (13) observed that the optimum pH for glutaminase activity of guinea pig brain tissue was between 8 and 9. That of guinea pig liver apparently occurred at a somewhat more acid reaction. Our results are substantially in agreement with Krebs.

Effect of Varying Phosphate Concentrations on the Desamidation of Glutamine with Rat Brain Extracts

With rat liver extract equivalent to 330 mg. tissue/cc., and with 1 hour of incubation at 37°C., the maximum effect of added phosphate on the desamidation of 14 micromoles of glutamine was reached at about 0.03 M concentration (1). The determination of this optimal phosphate concentration is rendered somewhat difficult by the relatively small but variable amount of inorganic phosphate naturally present in the tissue extract. Per cc. of rat liver extract, equivalent to 330 mg. of tissue, there may be an inorganic phosphate concentration ranging from 2–5 $\times$ 10^{-4} M. Similarly, for rat brain the range may be 1–3 $\times$ 10^{-4} M. If the values for added inorganic phosphate are corrected by 1 $\times$ 10^{-4} M, the effect of phosphate concentration on the desamidation of glutamine with rat brain extracts may be described by the curve in Fig. 2.
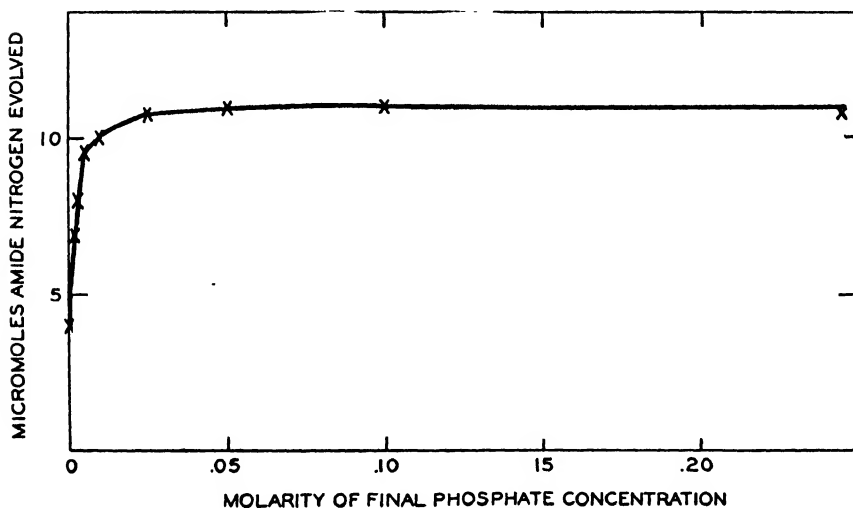
FIG. 2. Relation of final phosphate concentration to the desamidation of glutamine. Digests consisted of 1 cc. fresh aqueous rat brain extract, plus 2 cc. glycine-NaOH-NaCl buffer containing varying quantities of phosphate, plus 1 cc. of either water or 0.014 M glutamine. Initial and final pH of each digest 8.7 $\pm$ 0.05. Period of incubation 1 hour at 37°C.

As in the case of liver, the maximum concentration of phosphate is about 0.03 M, or at about a ratio of 2 moles of phosphate/mole of glutamine. No further effect is noted with higher concentrations of

phosphate. Complete desamidation of 14 micromoles of glutamine, as revealed by a 1 minute period of hydrolysis in boiling 1 N HCl, yields 13.5–13.7 micromoles of amide nitrogen (cf. 2). The extraordinary effect of relatively small quantities of added phosphate is noteworthy.

Effect of Added Anions on Various Amides with Extracts of Rat Liver and Rat Brain

The effect of adding phosphate, arsenate, sulfate, and pyruvate to digests of various amides with extracts of rat liver and of brain is described in Table II.

TABLE II

Effect of Anions on Desamidation of Amides in Digests with Rat Liver and Brain[a]

Amide	Micromoles $\times$ 10 ammonia N evolved in digests with									
	Liver in the presence of					Brain in the presence of				
	Buffer alone	PO₄	AsO₄	SO₄	Pyru-vate	Buffer alone	PO₄	AsO₄	SO₄	Pyru-vate
L-Glutamine[b]	20	110	90	88	48	22	100	98	90	20
L-Asparagine[b]	80	80	78	78	112	0	0	0	0	0
L-Isoglutamine[b]	100	100	100	100	100	16	18	17	16	16
Glycine amide[c]	20	22	20	18	20	2	3	3	2	2
DL-Alanine amide[c]	42[e]	42[e]	40[e]	44[e]	44[e]	19	18	17	18	18
DL-Leucine amide[c]	63[f]	64[f]	60[f]	65[f]	64[f]	98	103	100	98	100
L-Benzoylarginine amide[d]	76	22	8	70	76	14	4	3	12	14

[a] Digests consisted of 1 cc. of fresh aqueous extract equivalent, except where otherwise noted, to 330 mg. tissue/cc., plus 2 cc. of either veronal acetate-NaCl or glycine-NaOH-NaCl buffer with and without added anions, plus 1 cc. of either water or substrate solution. Final concentration of added anions 0.02 M, except for benzoylarginine amide which was 0.05 M. Period of incubation 1 hour at 37°C. except for benzoylarginine amide, which was 4 hours. Stock substrate solutions were 0.014 M for glutamine, asparagine, and isoglutamine, 0.025 M for all others. Stock solutions of the amides were brought to pH 7.0 by addition of dilute HCl.

[b] Beginning and final pH 8.2 $\pm$ 0.05.

[c] Beginning and final pH 8.6 $\pm$ 0.05.

[d] Beginning and final pH 6.2 $\pm$ 0.02.

[e] Extract concentration equivalent to 33 mg. fresh tissue/cc.

[f] Extract concentration equivalent to 4 mg. fresh tissue/cc.

The desamidation of the amino acid amides studied, *i.e.*, isoglutamine, glycine amide, alanine amide, and leucine amide, is apparently

unaffected by the presence of any one of the added anions. The desamidation of asparagine is increased by pyruvate in extracts of liver, but not by any of the other anions studied. Glutamine, however, is desamidated more rapidly in extracts of liver, brain, and spleen (*cf.* Table I) in the presence of added phosphate, arsenate and sulfate, whereas benzoylarginine amide is desamidated more slowly in such extracts in the presence of added phosphate and arsenate (but not of added sulfate or pyruvate).

Effect of Phosphate Concentration on the Desamidation of Benzoylarginine Amide

The effect of adding increasing amounts of phosphate to digests of benzoylarginine amide with rat liver extracts is shown in Fig. 3.
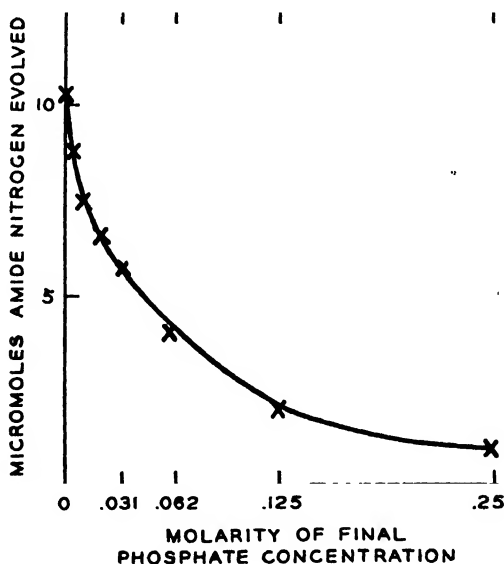
FIG. 3. Relation of final phosphate concentration to the desamidation of benzoylarginine amide. Digests consisted of 1 cc. fresh aqueous rat liver extract, plus 2 cc. veronal acetate-NaCl buffer containing varying quantities of phosphate, plus 1 cc. of either water or 0.025 M substrate. Initial and final pH of each digest 6.2 ± 0.05. Period of incubation 4 hours at 37°C.

Great care was taken to set the pH of each digest at 6.2. The pH at the end of the digestion period did not vary from this initial value by more

than 0.05. With increasing concentration of phosphate, the activity toward the substrate decreases considerably.

Effect of pH on the Desamidation of Benzoylarginine Amide in the Presence of Added Phosphate

Fig. 4 illustrates the fact that the relation of pH to the desamidation of benzoylarginine amide is the same in the presence of added phos-
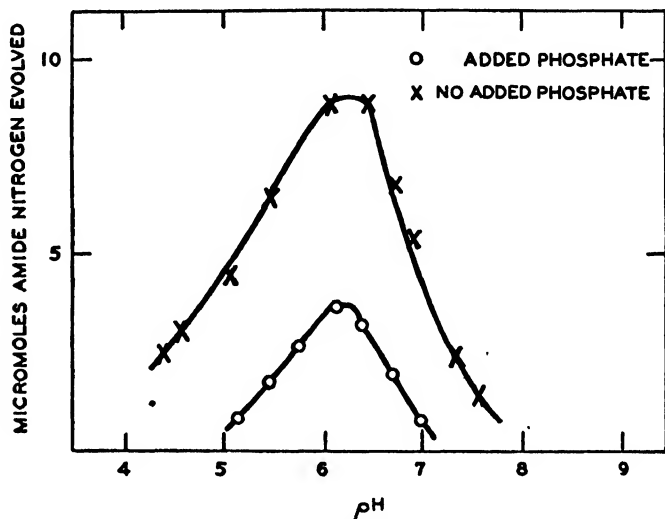

FIG. 4. Relation of pH of the digest to the desamidation of benzoylarginine amide in rat liver extracts with and without added phosphate. Digests consisted of 1 cc. fresh aqueous rat liver extract, plus 2 cc. veronal acetate-NaCl buffer with and without added phosphate, plus 1 cc. of either water or 0.025 M substrate. Final concentration of added phosphate 0.05 M. Period of incubation 4 hours at 37°C. Initial and final pH values varied by no more than 0.05.

phate as in its absence. The pH optimum of 6.2 is the same as that reported earlier (9).[3]

DISCUSSION

The striking effect of various added anions on the enzymatic desamidation of certain susceptible amides in tissue extract preparations

[3] The pH optimum for benzoylarginine amidase activity in a beef spleen preparation was reported by Fruton and Bergmann (14) to be about 4.7. It is difficult to compare their findings with ours, since Fruton and Bergmann were working with an acidified and isolated protein fraction from beef spleen, whereas we have utilized the complete rat liver extract.

is noteworthy. Pyruvate accelerates the desamidation of glutamine and asparagine in digests of liver, but has no effect in digests of other tissues studied. Phosphate, arsenate, and sulfate accelerate the desamidation of glutamine, but not of asparagine, in digests of liver, spleen, and brain. Whereas phosphate and arsenate inhibit the desamidation of benzoylarginine amide, pyruvate has no effect.

Further differences in the effect of pyruvate and of phosphate on the desamidation of glutamine in liver digests are (a) the optimal pH at which glutamine is desamidated, with or without added phosphate, is about 8.1 (1, 6), whereas, in the presence of pyruvate, the optimal pH is about 7.0 (6); (b) the accelerating effect of phosphate is destroyed if the digest is acidified below pH 5.0, or heated at 50°C., whereas the accelerating effect of pyruvate is relatively unaffected (6); and (c) increase in pyruvate concentration beyond a ratio of 2–3 moles per mole of substrate results in a decrease in the effect (11), whereas a similar increase in phosphate concentration results in no apparent further change (Fig. 2).

It would appear, therefore, that the effects of pyruvate and phosphate belong to different enzyme reactions. Furthermore, the kidney glutaminase which is unaffected by polyvalent anions is probably different from the liver (or the brain and spleen) enzymes, a suggestion which had been advanced earlier by Krebs (13). There may thus be three kinds of tissue glutaminase.

It has been suggested that the effect of pyruvate on the desamidation of glutamine or asparagine in liver digests is accomplished (a) through the condensation of amide with keto acid to form a dehydropeptide, which is then (b) hydrolyzed by dehydropeptidase to yield ammonia, glutamic acid, and the regenerated pyruvic acid (2–7). Glutaminase or asparaginase activity is presumably not concerned in these phenomena involving pyruvate. On the other hand, the effect of the polyvalent inorganic anions on the desamidation of glutamine in digests of liver, brain, or spleen is most probably concerned with the glutaminase of these tissues. Whether such anions form a labile intermediate with glutamine, which is highly susceptible to the action of a particular glutaminase, or whether they act directly upon the enzyme, awaits further study. Whatever the explanation may be for the effect of pyruvate or the polyvalent anions on the acceleration of desamidation, it is clear that this effect seems to be restricted to ω amides, for such α amides as isoglutamine or leucine amide were unaffected (Table II).

Phosphate and arsenate can, however, *decrease* the desamidation of such an α amide as benzoylarginine amide.

The phenomena noted and the explanations advanced are based upon preliminary studies with aqueous tissue extracts. Further studies will be concerned with purified tissue fractions.*

SUMMARY

The effect was studied of added phosphate, arsenate, sulfate, nitrate, ethylphosphonate, methylarsonate, benzenesulfonate, pyruvate, and pyruvoylglycinate, on the desamidation of glutamine in aqueous extracts of various rat tissues. Phosphate, arsenate, sulfate, and ethylphosphonate accelerated the desamidation of the substrate in extracts of liver, brain, and spleen, but not in extracts of kidney. Pyruvate accelerated the desamidation only in liver. The optimum of 8.6 is obtained in digests of glutamine with brain extracts with or without added phosphate. The maximum effective concentration of phosphate is reached at a ratio of about 2 moles of phosphate per mole of glutamine.

Phosphate, arsenate, and sulfate have no effect on the desamidation of asparagine, isoglutamine, glycine amide, alanine amide, or leucine amide. Phosphate and arsenate produce a marked inhibition of the capacity of tissue extracts to hydrolyze benzoylarginine amide, the optimum pH for benzoylarginine amidase activity, 6.2, being the same in the presence and absence of added phosphate.

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Partial Hydrolysis of Human Hair

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INTRODUCTION

The products of partial hydrolysis of human hair under mild conditions seem to have received little study. Hair has usually been employed as a raw material for the preparation of certain amino acids, cystine, tyrosine, and sometimes arginine, after complete acid hydrolysis. Because of their extreme insolubility, hair and other keratins are not amenable to enzymatic hydrolysis, usually employed with other proteins for production of partial hydrolysis products. However, acid hydrolysis at lower temperatures proceeds at greatly reduced speed, thus making possible the isolation of intermediate products. Moreover, the course of hydrolysis is somewhat dependent on the identity of the acid employed. One German patent (1) records a process by which keratins are partly hydrolyzed, using higher temperatures and higher concentrations of acid than we have employed.

In the present studies, hydrolysis was carried on at 38°C. by means of 3 M H_2SO_4 used in the proportion of 2 ml. of this acid/g. of defatted, washed and dried hair.

In comparison with the effect of other acids of equivalent concentration used under the same conditions, H_2SO_4 is unique in that the visible change in the gross appearance of the hair is very slight. For example, in two weeks time, the hair immersed in the H_2SO_4 looked the same as at the beginning, whereas in HCl it was completely disintegrated and in H_3PO_4 the same was true, a very thick gelatinous mass having been formed. In H_2SO_4 the hair retains the same physical appearance even when kept for a period of years under these conditions. However, results of various such experiments have shown that there is a gradual extraction of protein from the hair accompanied by a gradual darkening of the acid layer. This layer has given a very strongly positive biuret test, at any time from about 4 months to over 4 years. During such periods of time the properties of the dissolved protein have been shown to change very little, as indicated by analyses such as those described below. The longer periods of time merely produced a somewhat greater degree of extraction of the protein of the hair. The results described below were those obtained from a lot of hair which had stood in contact with the acid at 38°C. for 1240 days.

EXPERIMENTAL

Methods

The chief qualitative tests employed were the biuret and nitroprusside (with sodium cyanide) for cystine.

Determinations of total nitrogen were made by the Kjeldahl procedure. Free amino nitrogen was determined by Sörensen formaldehyde titration, confirmed, in some cases, by the Van Slyke method.

Because of the large quantity of sulfuric acid present, determinations of non-sulfate sulfur were preceded by removal of all but a small amount of the sulfate. For this purpose an aliquot of the solution under examination was treated with an amount of barium chloride slightly less than that required for complete precipitation of the sulfate. The barium sulfate was removed by centrifuging and washed 3 times. Filtrate and washings were made up to volume and aliquots of this solution were used for determinations of total sulfur by Parr bomb, and of total remaining sulfate. The difference between these was calculated as non-sulfate sulfur and contained both the cystine combined in the protein and its oxidation product (cysteic acid). Free cystine was never found in the protein solutions (negative Sullivan test). Combined cystine was determined after complete hydrolysis by boiling in acid, using the Folin-Marenzi (2) procedure, supplemented, in some cases, by the Sullivan method (3). For reasons detailed below the difference between the non-sulfate sulfur and the cystine sulfur was regarded as cysteic acid, resulting from slow oxidation of liberated cystine, since air was not excluded from the hair-acid mixtures during the intervening months and years.

The optical activity of the protein hydrolyzates was determined in a Schmidt and Haensch polarimeter with sodium vapor lamp, reading to $\pm 0.01°$. The results were arbitrarily calculated in terms of the specific rotation of the protein, the concentration of the latter being taken as $N \times 6.25$. As will be noted below, the total nitrogen figure, as well as those for free amino nitrogen and optical activity, require correction because of the presence of cysteic acid.

The chief example of the use of the above methods was the case of the hydrolysis of 3 kilos of washed and dried human hair in 6 l. of $3 M \ H_2SO_4$. After 1240 days standing at 38°C., the undisintegrated hair was filtered and sucked dry as possible on a Buchner funnel. This filtrate amounted to only 2900 ml.—less than half of the acid originally added. The residual hair was then washed by agitation with 30 separate washings, each of about 500 ml. water. After each washing the hair was filtered and sucked dry. The combined washings amounted to 14,170 ml. They were analyzed separately from the original filtrate. The residual hair was then washed as before with 18 separate washings of 500 ml. each of 95% alcohol. The total volume of these amounted to 8850 ml. The alcohol washings were also analyzed separately.

The original filtrate of 2900 ml. was the chief object of examination but no data resulting from analysis of the water or the alcohol washings indicated any marked difference in the proteins of these three solutions. In all cases the biuret test was strongly positive. The nitroprusside-

cyanide test for —S—S— linkages was reasonably positive and the Sullivan test for free cystine completely negative. A portion of the original filtrate neutralized to pH 4 produced a negligibly small precipitate, even after standing for several days at 0°C.

TABLE I

Analysis of Partial Hair Hydrolyzate, both Uncorrected and with the Protein Figures Corrected for the Presence of Cysteic Acid

Constituent	Filtrate (2900 ml.)		Water washings (14,170 ml.)		Alcohol washings (8850 ml.)
	Uncorrected	Corrected	Uncorrected	Corrected	Uncorrected
Total nitrogen (g.)	124.8	116.3	143.3	137.8	7.40
Nitrogen × 6.25 (g.)	780.0	727.0	895.0	860.0	46.25
Per cent of hair used	26.0		29.8		1.5
Amino nitrogen (g.)	53.8	45.3	58.7	53.2	3.26
Per cent amino to total nitrogen	43.1	39.0	41.0	38.6	44.1
Non-sulfate sulfur (g.)	40.9		37.6		1.31
Apparent per cent of S in protein	5.24		4.20		2.83
Cystine S (g.)	21.44		24.90		1.25
Per cent cystine S in protein	2.75	2.95	2.78	2.90	2.70
$[\alpha]_D^{25}$ (1 dm.)	−13.0		−2.86		−0.26
$[\alpha]_D^{25}$ based on protein	−48.3	−53.2	−45.2	−47.8	−49.7

Table I shows the analytical results obtained from the filtrate, the water washings, and the alcohol washings. The first column of figures are those uncorrected for the cysteic acid present. In addition, there are inserted, for the filtrate and water washings, figures showing the effect of this correction, made on the assumption that all non-sulfate sulfur is present either as combined cystine or as cysteic acid. It will be noted that the total amount of protein accounted for on the basis of the nitrogen (1721 g.), amounts to only a little over half of that initially used, and the ratio of free amino to total nitrogen is what might be expected from a mixture of peptones. The sulfur figures are noteworthy. Whereas the total non-sulfate sulfur in filtrate and water washings approximates that of the original hair, that of the alcohol washings is much lower and compares closely with the percentage of

cystine sulfur as determined by the Folin-Marenzi procedure in the protein of all three solutions. This percentage shows remarkable constancy at an average value of about 2.75%, or 2.92% if corrected, higher than that recorded for most proteose-peptone mixtures but decidedly lower than that of the original hair. Such a figure indicates that a large proportion of the originally combined cystine must have been separated in the course of formation of the proteose-peptone. Most investigators concur in assigning practically all of the sulfur in most animal hairs investigated to cystine (4, 5, 6), although Beveridge and Lucas (7) report the separation of nearly 1% methionine from human hair. However, the preponderance of evidence in this regard seems to justify our present assumption that any methionine involved is too small to be considered.

As shown by Andrews, Cornatzer and Sample (8), emulsification with chloroform can be used, under proper conditions, as a fairly efficient method of precipitating proteins of high molecular weight from solution. This procedure, however, causes very little precipitation of proteose-peptone mixtures, and the above authors reported removal of about 3–7% of the protein of such commercial preparations as Witte and Difco proteose-peptone. It is not surprising, therefore, that this treatment, when applied to these partial hair hydrolyzates, gave only a negligibly small precipitate.

Fractionation of the protein in the original filtrate by the methods of Wasteneys and Borsook (9) showed that less than 5% was precipitated by tricholoroacetic acid. Saturation with Na_2SO_4 at 33°C., and analysis of the filtrate from this precipitation, showed that 36% of the protein was precipitated as the proteose fraction, leaving the remainder as peptone. Sulfur and sulfate determinations made on the filtrate by the method described above showed that the precipitated proteose contained only about 1.8% sulfur. Folin-Marenzi determinations of cystine sulfur in hydrolyzed aliquots of the filtrate gave figures averaging 3.02% sulfur in the peptone. These values would calculate to about 2.6% cystine sulfur in the whole proteose-peptone mixture, a figure comparable to the 2.75% recorded for cystine sulfur in the protein of the filtrate (Table I). The non-sulfate, non-cystine sulfur fraction had largely passed into the filtrate containing the peptone.

The behavior of the constituents of the filtrate on dialysis was also investigated. 200 ml. were dialyzed through a collodion bag suspended in 500 ml. water. This

dialysis, with protection against bacterial action by means of toluene, was continued for 3 weeks, after which separate determinations of total nitrogen, amino nitrogen, non-sulfate sulfur, cystine sulfur, and optical activity were made.

The results for both the interior and the exterior solution are shown in Table II. While this procedure did not accomplish any quantitative

TABLE II

Dialysis of Proteose-Peptone from Hair

Two hundred ml. filtrate used. Data both uncorrected and with the protein figures corrected for the presence of cysteic acid

Constituent	Interior solution		Exterior solution	
	Uncorrected	Corrected	Uncorrected	Corrected
Total nitrogen (g.)	4.715	4.553	3.948	3.537
Nitrogen × 6.25 (g.)	29.5	28.5	24.7	22.1
Per cent of original protein	54.8		45.9	
Amino nitrogen (g.)	1.925	1.763	2.174	1.763
Per cent amino to total nitrogen	40.8	38.7	55.1	49.8
Total non-sulfate sulfur (g.)	1.16		1.64	
Per cent non-sulfate sulfur	3.94		6.64	
Cystine sulfur (g.)	0.79		0.70	
Percent cystine sulfur in protein	2.68	2.77	2.84	3.17
$[\alpha]_D^{25}$ based on protein	−51.7	−54.0	−35.6	−41.8

separation of the proteins in the mixture, it did serve to add further evidence that the non-sulfate sulfur consists of at least 2 forms: the cystine sulfur combined in the protein, and a separate fraction, most probably cysteic acid, resulting from atmospheric oxidation, during the long standing of the solution, of cystine split out. The percentage of cystine sulfur, based on the protein content as shown by Kjeldahl determinations, again shows a rather remarkable degree of constancy, whereas that part of the non-sulfate sulfur not accounted for as cystine was washed out into the outer chamber and was found to be somewhat roughly partitioned in the proportion of the volumes of the inner and outer solutions (2 to 5). As a result, the percentage of *total* non-sulfate sulfur based on the protein, varies widely as shown in both Table I and Table II, and is of no significance, whereas that of the bound cystine is satisfactorily constant.

Since the cysteic acid, most of which was dialyzed into the outer

solution, exerts in any of these solutions its effect on the figures for total nitrogen, amino nitrogen, non-sulfate sulfur, and optical activity, it is of interest to note the direction in which these figures have probably been distorted, and the basis for the corrected figures which are included in both tables.

Considered qualitatively, the following adjustments might be made: The protein content is too high, having been calculated from the total nitrogen, which includes the cysteic acid. The optical activity based on this protein figure is, therefore, too low, since the activity of the cysteic acid (10), although small (+9.0), is in the opposite direction to that of the protein and a smaller actual amount of protein is therefore responsible for a higher levorotation. The apparent ratio of amino to total nitrogen is somewhat too high since, for the cysteic acid present, this ratio is unity.

Calculation of the quantitative effect of these adjustments may best be made on the basis of the figures in Table I for the original filtrate (2900 ml.) and for the washings (14,170 ml.) of that lot of hair. Such calculations are based entirely on the assumption that all non-sulfate and non-cystine sulfur may be considered to be cysteic acid. While quantitative proof of such an assumption is lacking, the abundant presence of cysteic acid may be regarded as demonstrated by the following:

A 200 ml. sample of the dialyzate described above was evaporated slowly to dryness. The proteose-peptone mixture with the free sulfuric acid remained as an amorphous sticky residue in which were imbedded a number of fair sized crystals. These were picked out mechanically, redissolved in a small amount of water and recrystallized 3 times. About 0.5 g. of crystals resulted. These gave the following analytical figures: Calculated for cysteic acid: N, 8.27%; S, 18.93%. Found: N, 8.12%; S, 18.44%.

The conclusion seems justified, therefore, that at least a considerable portion of the sulfur of the protein was, at some stage, oxidized to cysteic acid. Although atmospheric oxidation of cystine to cysteic acid has been found previously to take place more readily in hydrochloric than in sulfuric acid (10, 11), these results were obtained in solutions containing only cystine, the acid, and traces of heavy metals. The variety of constituents in the solution obviously makes the conditions of the present experiment quite different.

Assuming, then, that all non-sulfate sulfur was either present as cystine combined in the protein (negative Sullivan reaction) or as free

cysteic acid, we may apply this correction to the total contents of the 2900 ml. of filtrate.

Cysteic acid sulfur = 40.90–21.44 = 19.46 g.

This is equivalent to 102.7 g. cysteic acid and to 8.5 g. cysteic acid nitrogen.

Protein nitrogen = 124.8–8.5 = 116.3 g.

Protein = 116.3 × 6.25 = 727 g.

Amino nitrogen = 53.8–8.5 = 45.3 g.

Per cent amino to total nitrogen = 45.3/116.3 × 100 = 39.0%.

Per cent cystine sulfur in the protein = 21.44/727 × 100 = 2.95%.

Optical activity:

102.7 g. cysteic acid/2900 ml. = 3.54 g./100 ml.

Assuming $[\alpha]_D$ of cysteic acid = +9.0 under these conditions, this accounts for an observed rotation in a 1 dm. tube of $9.0 × 3.54/100 = +0.319°$.

Applying this correction to the observed rotation of −13.0, the latter, for the protein alone, is raised to −13.32°.

The corrected protein content = 727. g./2900 ml., or 25.06 g./100 ml. $[\alpha]_D^{25} = 100 × 13.32/25.06 = −53.2$.

Applying the same corrections to the total contents of the water washings (14, 170 ml.) we obtain the corrected results which, together with those of the filtrate and the solutions resulting from dialysis, are summarized in Tables I and II.

The behavior of the constituents of this filtrate toward tryptic hydrolysis was also investigated. A sample of the original filtrate was freed of most of the sulfuric acid present by treatment with slightly less than its equivalent of $BaCl_2$. The filtered solution was neutralized to pH 7.5. Total nitrogen and formaldehyde determinations on aliquots of this gave a figure of 41.9% free amino to total nitrogen. Two hundred ml. of this solution were treated with 2 g. dry commercial trypsin and progressive formaldehyde titrations were made up to 96 hours. During this time the percentage of free amino to total nitrogen increased in the normal way to 59.6%. Complete acid hydrolysis of another aliquot raised this figure to 65%. It is obvious, therefore, that this product is susceptible to tryptic hydrolysis.

Insoluble Residue from Hair Hydrolysis

The insoluble residue after repeated washings with alcohol (see above) was dried. While retaining the superficial appearance of hair, both to the naked eye and under microscopic examination, it was extremely brittle and was easily reduced to a gray powder of which a total of about 1200 g. was obtained.

The following determinations were made on samples dried to constant weight *in vacuo*.

Total N (by Kjeldahl), 11.91%; total S (Parr bomb), 5.20%.

Complete hydrolysis by refluxing 15 hours with 20% HCl produced a dark brown solution with a large amount of insoluble residue. The solution was clarified with decolorizing carbon and tested for cystine by both the Folin-Marenzi and Sullivan procedures. In both cases the results were negative.

Several repetitions of hair hydrolysis with sulfuric acid under the above described conditions gave closely comparable results. In all cases 6 N H_2SO_4 was used in an amount double the weight of the hair. In one case, after standing for only 120 days at 38°C., the filtrate and washings from the undisintegrated hair contained about 45% of the protein originally added in the form of hair. The specific rotation of this solution, in terms of protein, and uncorrected for cysteic acid, was -48.5, a close duplicate of the figure reported above. The sulfur content of the protein and its other properties, including digestibility by trypsin, were practically identical. However, the non-sulfate sulfur content of the protein was, in all cases, far above what could be accounted for on the basis of cystine determinations. In no case could any free cystine be demonstrated by the Sullivan procedure, and Folin-Marenzi determinations, after complete acid hydrolysis, accounted for 40–50% of the non-sulfate sulfur. Since the hydrolyses were carried on with access of air it may be assumed that any liberated cystine, not an integral part of the proteose-peptone mixture, was oxidized to the sulfonic acid. Cysteic acid, being highly water soluble, would obviously accompany the filtrate and water washings of the hair and would dialyze easily through a collodion bag. Such results might be taken to indicate that the mechanism of this hydrolysis involves a preferential affinity for at least part of those peptide linkages in which the cystine participates.

SUMMARY

Slow hydrolysis of human hair by means of 3 M H_2SO_4 at 38°C. produces a solution of proteose-peptone with a free amino to total nitrogen ratio of about 39%, a cystine sulfur content of about 2.9%, and an optical activity, calculated to the protein, of about -50.

In spite of variations of from 4 months to 4 years in the time of hydrolysis, the product obtained shows considerable constancy as regards the above figures.

The total non-sulfate sulfur of such solutions is about twice the cystine sulfur. The evidence indicates that the non-sulfate, non-cystine sulfur is partly, if not all, in the form of cysteic acid. The cysteic acid appears to have resulted from atmospheric oxidation of a part of the cystine, whereas that part of the latter remaining un-oxidized is contained in the proteose-peptone.

The proteose-peptone is susceptible to digestion by trypsin.

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Bone Marrow for Fat Storage in Rabbits

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INTRODUCTION

The marrow cavities of the bones afford space for the storage of substantial reserves of lipides. This storage is unique, inasmuch as such fat does not increase the bulk of the animal body. Little attention has been given to the movement of fats in and out of the marrow cavities. In 1898 Winternitz (1) found iodized fats in the marrow of dogs after such fats had been fed. In 1930 Milbradt (2) increased the content of marrow cholesterol by feeding this substance to rabbits.

The purpose of the present study was to determine the effect of the quality of dietary fat upon that deposited in bones.

EXPERIMENTAL

Young adult rabbits were used as experimental animals.

The basal diet fed was a mixture of alfalfa meal 69, crushed oats 23, ground cellophane 4, agar 3, and bone meal 1.

Two series of experiments were run using 14 rabbits. Representative animals were killed at the end of each of a series of periods. The distribution of lipides in various tissues, as well as the iodine numbers of these lipides, were determined.

In the first series during the first period of 11 days rabbits were maintained upon the stock diet. During the second period, food intake was restricted so that they lost 20% of their body weight in the course of 12 days, and depleted their fat reserves. In the third period of 14 days these stores were replenished by liberal feeding of cottonseed oil to provide 55% of the calories of the diet. In the final period of 14 days the amount of the diet was restricted. The lipides of the basal diet had an iodine number of 91 while the supplement raised this to 101.

During each period the feed and feces were analyzed for lipides to determine the amount utilized, since little is known about fat absorption by rabbits.

Conventional methods were employed except that an alcohol-ether mixture was used for the extraction of lipides of the dried left humerus and right femur. These

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were selected as typical bones with a large marrow capacity. Iodine numbers were run by the Rosenmund-Kuhnhenn (3) method.

RESULTS

The data of Table I indicate that the adult rabbit can consume more than half of its calories in the form of a low melting fat without serious disturbance.

TABLE I

Feed Intakes and Fat Digestibilities

Experimental period and number of rabbits killed at end of each		Amount of basic diet consumed daily by each rabbit	Per cent fat in feed	Total daily calorific intake per rabbit	Per cent fat digested	
		g.		*cal.*		
First Feeding Experiment						
I.	Maintenance (1)	75	2.1	167	63	
II.	First restricted					
	calorie (1)	30	2.1	67	71	
III.	High fat (2)	45+15 ml. cottonseed oil[a]	24.4	223.	After one week:	78
					After two weeks:	86
IV.	Second restricted					
	calorie (2)	30	2.1	67	After one week:	43
					After two weeks:	61
Second Feeding Experiment						
I.	Maintenance (1)	75	2.1	167	66	
II.	First restricted					
	calorie (1)	30	2.1	67	72	
III.	High fat (3)	30+15 ml. cottonseed oil	32.1	180	After four days:	85
					After one weeck:	92
					After two weeks:	87
IV.	Second restricted				After one week:	17
	calorie (3)	30	2.1	67	After two weeks:	61
					After three weeks:	57

[a] 15 ml. = 13.2 g.

The utilization of this fat varied from 78 to 92% in spite of the high level consumed. No correction was made for metabolic lipides nor for calcium soaps since this was not the primary purpose of the study.

The values for the iodine numbers of lipides from bones, after feeding cottonseed oil, indicate that bone lipides can be classified

among the stores such as the mesentery and subcutaneous fats (Table II).

TABLE II

Iodine Number Values of the Bone and Tissue Fats

Experimental period (days)		Bone	Liver	Omen-tum	Mesen-tery	Peri-renal	Subcu-tane-ous	Skel-etal muscle	Brain
First Feeding Experiment									
I. Maintenance	(11)	84	40	82	70	80	77	60	—
II. Restricted	(12)	76	43	45	50	55	46	80	—
III. High fat	(7)	101	72	100	103	110	102	78	—
High fat	(14)	107	66	104	106	106	104	77	62
IV. Restricted	(7)	95	81	102	102	111	113	72	73
Restricted	(14)	100	50	72	65	69	100	69	64
Second Feeding Experiment									
I. Maintenance	(12)	89	40	85	80	82	83	61	65
II. Restricted	(12)	88	47	77	79	81	78	44	65
III. High fat	(4)	87	52	65	75	70	71	73	62
High fat	(7)	102	62	103	103	104	106	64	61
High fat	(14)	109	60	108	109	109	111	66	64
IV. Restricted	(7)	102	53	106	111	109	105	65	62
Restricted	(14)	92	42	106	102	106	99	56	63
Restricted	(21)	101	40	55	58	56	53	59	64

After the periods of restriction, the iodine numbers of the bone lipides attained levels from feeding the unsaturated oil, similar to those in the mesentery. However, the decline of these values during restriction of food intake after high fat feeding was slower, indicating that bones surrender fats more slowly.

The level of bone lipides indicated in Table III shows the fluctuations to which bones are subjected when a rabbit passes from restricted to excess food in the course of a couple of months.

Thus, at the end of the high fat period in the first series, the bones yielded 30% lipides while two weeks of restricted feeding reduced this value to half.

In the second study, by extending the restricted period to 3 weeks, a value as low as 3% was found.

Such variations were familiar to the plains Indians who differentiated sharply in their food preferences between bone marrow of the buffalo with much fat and with little.

TABLE III

Bone Fat Values

Ether-alcohol soluble fraction, average for left humerus and
right femur, dry weight basis

First Feeding Experiment	Per cent
1. End of maintenance period (11 days)	16.4
2. End of first calorific restriction (12 days)	10.5
3. High fat period (7 days)	15.9
4. End of high fat period (14 days)	30.3
5. Second calorific restriction (7 days)	13.8
6. End of second calorific restriction (14 days)	14.2
Second Feeding Experiment	
1. End of maintenance period (12 days)	19.2
2. End of first calorific restriction (12 days)	16.4
3. High fat period (4 days)	1.5
4. High fat period (7 days)	9.3
5. End of high fat period (14 days)	19.9
6. Second calorific restriction (7 days)	17.7
7. Second calorific restriction (14 days)	21.7
8. End of second calorific restriction (21 days)	3.3

Thus, marrow provides the animal body with a unique storage space
for reserve nutrients without increase of bulk of the body.

SUMMARY

The marrow cavities in the bones of rabbits serve as storage space
for mobile supplies of lipides. The iodine number of these fats reflects
that of the diet if the fat stores are depleted and then flooded with an
unsaturated lipide. Rabbits can be fed diets in which 55–63% of the
calories are included as cottonseed oil. Of this fat 78–92% is absorbed
by various rabbits.

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The Inhibition of Hexokinase by Amidone (2-Dimethyl-amino-4,4-Diphenylheptanone-5-Hydrochloride) [1]

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INTRODUCTION

In an attempt to elucidate the mechanism of action of narcotics, we have been investigating the effects of these drugs on the metabolism of carbohydrate in brain. There is considerable evidence that anaesthetics inhibit certain oxidative process essential for the metabolism of carbohydrate (1, 2, 3). Since amidone (2-dimethylamino-4, 4-diphenylheptanone-5), morphine and demerol (ethyl 1-methyl-4-phenylpiperidine-4-carboxylate) all possess marked analgesic properties but are reputed to differ in their sedative or narcotic effect, it was decided to compare the effects of these 3 drugs on carbohydrate metabolism in rat brain, and to try to determine wherein they might resemble or differ from the anaesthetics. The results of experiments presented in this paper show that amidone differs from morphine and demerol in its effect on glycolysis in both rat brain and frog muscle. Evidence is also presented to indicate the site in the glycolytic pathway at which amidone exerts this effect.

METHODS

The glycolytic rate was measured manometrically, using Warburg manometers with vessels of about 15 cc. capacity, and a bicarbonate buffer.

Three different bicarbonate buffers were used for rat brain and will be designated as A, B, and C in the tables. Buffers A and B were used for whole brain. These differed mainly in their potassium and sodium content. The results of experiments in which these buffers were used, were, however, similar. Buffer C was used for brain extract. The composition of these buffers was as follows. Buffer A (4) contained 0.1185

M NaCl, 0.0047 M KCl, 0.0025 M CaCl$_2$, 0.0012 M KH$_2$PO$_4$, 0.0012 M MgSO$_4$, and 0.0249 M NaHCO$_3$. Buffer B (5) contained 0.025 M KHCO$_3$, 0.0752 M K$_2$HPO$_4$, and 0.01 M MgSO$_4$. Buffer C (6) contained 0.016 M NaHCO$_3$, 0.0035 M MgCl$_2$, 0.01 M NaK phosphate buffer,[2] pH 7.3, and 0.012 M nicotinamide. The buffer used for frog muscle contained 0.03 M NaHCO$_3$ and 0.05 M NaK phosphate buffer, pH 7.4.[2]

In experiments with whole brain, the tissue was homogenized in buffer and diluted so that 1 cc. contained about 300 mg. tissue. In each vessel 0.5 cc. homogenate was used in a final volume of 1.5 cc. In experiments with brain extract the brain was homogenized with 4 parts ice cold water and centrifuged. Of the supernatant fluid 0.3 cc. was added to each vessel. The final volume was 2 cc.

In experiments with frog muscle, this tissue was removed from the hind legs and placed immediately in ice. It was ground with carborundum and 1.5 parts water in an ice cold mortar and centrifuged. One cc. of the supernatant fluid was used in each vessel in a final volume of 2 cc.

The final concentration of substrate was 0.013 M in experiments with whole brain, 0.01 M (or 0.18% for glycogen) in experiments with brain extract, 0.02 M (or 0.4% for glycogen) in experiments with frog muscle extract.

In some experiments with brain extract, yeast extract was added as a source of coenzymes. In others adenosinetriphosphate (ATP) and cozymase were added. Yeast extract was prepared by heating 1 part of Fleischmann's baker's yeast[3] with 1 part of water at 100°C. for 5–10 minutes. It was then centrifuged and the supernatant fluid was used in the experiments.

ATP was prepared from rabbit muscle as the barium salt (7). It was converted to the sodium salt by treating with an equivalent amount of Na$_2$SO$_4$ in water solution and centrifuging.

Cozymase was prepared from Fleischmann's baker's yeast[3] by the method of Williamson and Green (8).

Fructose-6-phosphate was prepared by the method of Neuberg et al. (9).

Amidone[4] was used as the hydrochloride in these experiments. When calcium was present, the final concentration was 0.002 M.

The gas phase in all manometric experiments was 95% N$_2$, 5% CO$_2$.

Experiments with rat brain were carried out at 37°C., those with frog muscle at 20°C.

Differences of less than 10% between the values obtained in control experiments and those with amidone are not considered to be significant.

RESULTS AND DISCUSSION

Table I shows that amidone, in concentrations of 0.002 M, or less, inhibited glycolysis of glucose by rat brain homogenates and by frog muscle extracts; demerol, in similar or higher concentrations, either

[2] Equimolar solutions of disodium hydrogen phosphate and potassium dihydrogen phosphate were mixed in the proportion of 8 to 2 and diluted to give the concentrations indicated.

[3] Kindly supplied by Standard Brands, Incorporated.

[4] Kindly supplied by the Mallinckrodt Chemical Works.

TABLE I

Effect of Morphine, Demerol and Amidone on Glycolysis of Glucose

Drug	Molar concentration	Duration of expt.	Buffer	Mm.³ CO₂ evolved control	Mm.³ CO₂ evolved with drug	Per cent effect
		min.				
			Rat brain			
Morphine	0.0056	60	A + Ca"	123	115	−7
Demerol	0.0032	60	A + Ca		171	+39
Amidone	0.0026	60	A + Ca		45	−63
Morphine	0.0028	·60	A − Ca	129	135	+5
Demerol	0.0032	60	A − Ca		140	+8
Amidone	0.0026	60	A − Ca		36	−72
Morphine	0.0028	60	A + Ca	129	122	−5
Demerol	0.0032	60	A + Ca		158	+23
Amidone	0.0026	60	A + Ca		48	−63
			Frog muscle			
Amidone	0.0009	35		149	117	−22
Amidone	0.0019	35			86	−42
Morphine	0.0021	35			154	+3
Amidone	0.0009	60		324	270	−17
Amidone	0.0019	60			166	−49
Morphine	0.0021	60			340	+5
Morphine	0.0042	60			360	+11
Demerol	0.0100	60			330	+2
Amidone	0.0009	50		181	143	−21
Amidone	0.0018	50			121	−33
Demerol	0.0100	50			185	+2

ᵃ + Ca = Calcium added; − Ca = No calcium added.

accelerated glycolysis or had no effect, depending on the calcium concentration in the case of brain; and morphine, in the same or higher concentrations, had no effect.

In an attempt to determine the site in the glycolytic cycle at which amidone exerted its effect, cell-free glycolyzing extracts of brain and

of frog muscle were prepared, which, in contrast with intact cells, metabolize added glycogen and hexosephosphates. The effect of amidone on the metabolism of these substrates was compared with that on the metabolism of glucose. Although the rate of glycolysis of glucose in brain extract was higher than that in whole brain, as has been observed by other workers (10), amidone decreased the rate in both cases, the effect being somewhat greater in whole brain. Inasmuch

TABLE II

The Effect of Amidone on the Glycolysis of Glucose, Glycogen, Hexosediphosphate, and Fructose-6-Phosphate

Substrate added	Molar concentration of amidone	Tissue	Buffer	Duration of experiment	Mm.³ CO₂ evolved		Per cent effect
					control	amidone	
Rat brain				*min.*			
Glucose	0.0006	whole brain	A+Ca	60	127	150	+18
Glucose	0.0012	whole brain	A+Ca	60		100	−21
Glucose	0.0024	whole brain	A+Ca	60		57	−55
Glucose	0.0012	whole brain	A+Ca	90	212	137	−35
Glucose	0.0024	whole brain	A+Ca	90		54	−75
Glucose	0.0012	whole brain	B	60	116	64	−45
Glucose	0.0024	whole brain	B	60		49	−58
Glucose	0.0024	brain extract	C	45	295	222	−25
Glycogen	0.0024	brain extract	C	45	117	117	0
Glycogen	0.0024	brain extract	C	60	92	95	+3
Glycogen	0.0024	brain extract	C	125	168	160	−5
Hexosediphosphate	0.0024	brain extract	C	125	93	102	+10
Glycogen	0.0024	brain extract	C	60	175	180	+3
Glucose	0.0024	brain extract	C	60	275	147	−47
Hexosediphosphate	0.0024	brain extract	C	60	84	88	+5
Glycogen	0.0024	brain extract	C	60	117	111	−5

TABLE II—*Continued*

Substrate added	Molar concentration of amidone	Tissue	Buffer	Duration of experiment	Mm.^{3} CO_2 evolved		Per cent effect
					control	amidone	

min.

Frog muscle

Substrate added	Molar concentration of amidone	Tissue	Buffer	Duration of experiment	control	amidone	Per cent effect
None	0.0019	muscle extract		55	154	139	−10
Glucose	0.0019	muscle extract		55	142	30	−79
Glycogen	0.0019	muscle extract		55	361	360	0
None	0.0019	muscle extract		60	150	170	+13
Glucose	0.0019	muscle extract		60	132	74	−44
Glycogen	0.0019	muscle extract		60	352	360	0
None	0.0019	muscle extract		45	246	195	−21
Glucose	0.0019	muscle extract		45	227	150	−34
Glycogen	0.0019	muscle extract		45	220	311	+41
None	0.0019	muscle extract		45	221	143	−35
Glucose	0.0019	muscle extract		45	178	87	−51
Glycogen	0.0019	muscle extract		45	232	320	+38
None	0.0019	muscle extract		60	204	124	−39
Glucose	0.0019	muscle extract		60	158	100	−42
Glycogen	0.0019	muscle extract		60	410	460	+12
Glucose	0.0019	muscle extract		55	138	75	−46
Fructose-6-phosphate	0.0019	muscle extract		55	238	221	−7
Glucose	0.0019	muscle extract		50	181	121	−33
Fructose-6-phosphate	0.0019	muscle extract		50	185	174	−6
Glycogen	0.0019	muscle extract		60	358	377	+5
Glucose	0.0019	muscle extract		35	149	86	−42
Fructose-6-phosphate	0.0019	muscle extract		35	160	151	−6

In experiments in which brain extract was used yeast extract was added as a source of coenzymes.

as Meyerhof and Gcliazkowa (10) have recently shown that one factor contributing to the increased glycolytic activity of brain extracts over that of whole brain is the concentration of ATP in these preparations, the increased inhibition of glycolysis produced by amidone in whole brain in our experiments might be attributed to the lower concentration of ATP in this preparation, as will be discussed later. While amidone inhibited the glycolysis of glucose it had no effect on the glycolysis of glycogen, fructose-6-phosphate, or hexose diphosphate in either frog muscle or rat brain extracts (Table II). In some cases, the addition of amidone accelerated the rate of glycolysis of glycogen by muscle extract. These findings indicate that amidone exerted its inhibitory effect on that reaction which is peculiar to the metabolism of glucose, namely the phosphorylation of glucose to glucose-6-phosphate, which is catalyzed by the enzyme hexokinase.

Considerable variation was found in the glycolytic activity in different experiments in which brain extracts were used. This variation is probably due to variation in the yeast extract, which remained active for several days but which gradually lost its activity on standing in the refrigerator.

The glycolytic activity of frog muscle without added substrate was in some cases inhibited by amidone. In these cases it seems likely that some free glucose as well as glycogen was present in the tissue, as determinations of glucose in the extract, after $Ba(OH)_2$-$ZnSO_4$ precipitation, indicated the presence of considerable reducing material (equivalent to 1.3 mg. and 0.6 mg. glucose/cc. extract in two different experiments).

In some experiments the glycolytic rate in the presence of added glucose was less than it was when no substrate was added. This may be explained by the finding of Lehmann (11) that the addition of glucose depressed the metabolism of glycogen, the degree of depression depending on the concentration of glucose. In the experiments without added substrate, it is likely that a mixture of glycogen with a small amount of glucose was metabolized, whereas, with added glucose, the metabolism of glycogen was suppressed and glucose was metabolized exclusively. Under the conditions of our experiments, glycogen, when present in excess, was metabolized at a faster rate than glucose by frog muscle extract.

In experiments with cell-free brain extract in which yeast extract was added as a source of coenzymes, it was found that, by increasing

the amount of yeast extract, the inhibitory effect of amidone on the glycolysis of glucose could be decreased. Likewise, when yeast extract was added to whole brain, the glycolysis of which was inhibited by amidone, a decrease in inhibition was produced (Table III). That this

TABLE III

Effect of Varying Amounts of Yeast Extract on Inhibition of Glycolysis by Amidone

Brain preparation	Buffer	Molar concentration of amidone	Yeast extract added	Duration of experiment	Mm.^{3} CO_2 evolved		Per cent effect
					control	amidone	
			cc.	*min.*			
Whole brain	B	0.0024		60	98	49	−50
			0.5	60	99	68	−31
Whole brain	B	0.0024	—	70	90	63	−30
			0.5	70	87	83	−5
Brain extract	C	0.002	0.2	60	162	61	−62
			0.4	60	245	201	−18
			0.5	60	279	257	−8

decrease in inhibition is due, in part at least, to ATP was shown by the effect produced by adding varying concentrations of ATP to brain extract and to whole brain whose glycolysis was inhibited by amidone. In both cases ATP produced a decrease in the inhibition (Table IV). This indicates that amidone inhibits kexokinase by competing with ATP for the enzyme.

It was also observed that the presence of calcium ions decreased the effect produced by amidone, especially in the lower concentrations (Table V). When no calcium was added, low concentrations of amidone (0.0012 M) inhibited glycolysis, whereas, when calcium was added, this concentration of amidone tended to accelerate glycolysis. With higher concentrations of amidone (0.0024 M) the effect was not so marked. These effects will be investigated further.

SUMMARY

1. Amidone, unlike morphine and demerol, inhibited glycolysis of glucose by rat brain and by frog muscle.

TABLE IV

Effect of Varying Concentrations of ATP on the Inhibition
of Glycolysis of Glucose Produced by Amidone

Brain preparation	Buffer	Molar concentration of amidone	Molar concentration of ATP	Duration of experiment	Mm.³ CO_2 evolved		Per cent effect
					control	amidone	
				min.			
Brain extract	C	0.002	0.00025	45	37	18	−52
		0.002	0.0005	45	54	49	−11
Whole brain	A	0.0024	---	35	97	65	−33
		0.0024	0.0013	35	85	68	−20
		0.0024	0.0026	35	82	79	0
Whole brain	B	0.0024	· ·	60	138	82	−41
		0.0024	0.0013	60	107	103	0
Brain extract	C	0.002	0.00012	60	46	30	−32
		0.002	0.0005	60	68	54	−21
			0.0010	60	· 154	370	+140

Cozymase was also added in experiments with brain extract in a concentration to produce maximum effects.

TABLE V

Effect of Calcium on the Inhibition of Glycolysis
of Glucose by Brain Produced by Amidone

Ca	Molar concentration of amidone	Duration of experiment	Mm.³ CO_2 evolved		Per cent effect
			control	amidone	
		min.			
—	0.0012	60	183	114	−38
—	0.0024	60		39	−79
+	0.0012	60	123	144	+17
+	0.0024	60		63	−49
—	0.0012	60	129	101	−21
—	0.0024	60		36	−72
+	· 0.0012	60	129	149	+16
+	0.0024	60		48	−63

2. Amidone had no inhibitory effect on the glycolysis of glycogen, fructose-6-phosphate or hexosediphosphate.

3. These findings are compatible with the assumption that the enzyme inhibited is hexokinase.

4. Addition of ATP to brain reduced the inhibition of glycolysis produced by amidone.

5. Addition of calcium ions to the medium decreased the amidone-produced inhibition of glycolysis by brain, especially in the lower concentrations of amidone used.

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Note on "An Osmotic Diffusion Pump"

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In a recent paper,[2] Franck and Mayer described an osmotic diffusion pump which is capable of utilizing chemical free energy to transport either solvent or solute from one solution to another in a direction opposite to the natural diffusion tendency.

It appears that a slight error was made in the section dealing with "The Solute Diffusion Pump." The purpose of the present note is to point out this correction, and to mention certain conclusions based upon the corrected equations.

The equation immediately preceding Franck and Mayer's Eq. [44] may be verified as being correct. However, the substitution

$$\zeta^{-1} = Q^*/Q_a, \tag{1}$$

which is their Eq. [45], gives, instead of their expression for R, the following:

$$R = [2 + \zeta + \zeta^{-1}][1 + \alpha/y] - 1 - \zeta^{-1}. \tag{2}$$

The last term, $-\zeta^{-1}$, was omitted from their Eq. [44]. The omission of this term changes the conclusions significantly.

For R to be a minimum, it is now found that

$$\zeta = \sqrt{\frac{\alpha/y}{1 + \alpha/y}} \tag{3}$$

instead of $\zeta = 1$, as found by Franck and Mayer. Substitution of Eq.

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[2] Franck, J., and Mayer, J. E., *Arch. Biochem.* **14**, 297 (1947).

(3) into Eq. (2) gives, after a little algebraic manipulation,[3]

$$R_{min} = [\sqrt{1 + \alpha/y} + \sqrt{\alpha/y}]^2. \tag{4}$$

From Eq. (4) it is seen that when (α/y) equals zero, R_{min} equals unity. This represents a complete conversion of the expended free energy into gained free energy (*i.e.*, 100% "efficiency"). Moreover, when (α/y) equals zero,

$$\zeta = Q_a/Q^* = 0, \tag{5}$$

so that the most "efficient" transfer occurs when there is no cycling of solute a. This seems to be more or less self-evident.

In actual practice, it is most likely that $(\alpha/y) \neq 0$, so that $R_{min} > 1$. Also, the actual mechanisms may, perhaps, be somewhat better approximated by a mechanism of the sort

$$n\,a + c \rightarrow b, \tag{6}$$

where only a can diffuse through the membrane. This is in line with the possible nature of the reactions discussed in the article. However, when the analysis is carried out, the term (α/y) in the two-solute case is replaced by the sum of two similar terms, so that the conclusions are substantially the same as for the two-solute case.

For some values of (α/y), R_{min} has the following values:

$(\alpha/y) =$	0.10	0.25	0.50	1.00
$R_{min} =$	1.86	2.61	3.73	5.83

From this table it is clear that reasonably small values of R_{min} may be obtained with reasonable values of (α/y).

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[3] The form of Eq. (4) was suggested by Professor Mayer, to whom the author is indebted for some helpful correspondence.

The Maintenance of *L. casei* and *L. arabinosus* Cultures in the Lyophilized State [1]

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The maintenance of stock cultures in a highly viable state is important if the results of microbiological assays for vitamins and amino acids are to be reliable. However, in a laboratory where microbiological assays are used only periodically, the frequent transfer of cultures can be replaced by the storage of cultures dehydrated from the frozen state (lyophilized).

Naylor and Smith (1) have reviewed the literature concerning the preparation and storage of dehydrated non-spore-forming bacteria. They have studied the factors affecting the viability of lyophilized *Serratia marcescens* cultures. Their work indicated that suspension of the cells in a solution containing ascorbic acid, thiourea, NH_4Cl, and dextrin at pH 6 to 7 gave maximum survival after lyophilization, when the samples were stored in a high vacuum.

The work of Nymon *et al.* (2) indicated that, at least for short storage periods, the lyophile process could be applied to both *L. arabinosus* and *L. casei* cultures. This study of lyophilization has been extended to include a year's storage of the cultures, and some changes have been made in the preparation of the lyophilized cultures.

EXPERIMENTAL

Preparation of Lyophilized Cultures

The *L. arabinosus* and *L. casei* cultures were grown in a nutrient-rich medium (3) for 20–22 hours at 37°C. These cultures were then centrifuged and the supernatant was decanted. To the cells obtained from 10 ml. of broth, 0.5 ml. of sterile skim milk was added. One-tenth ml. quantities of this milk suspension were pipetted into

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sterile, cotton-plugged vials (10 × 42 mm.). A very narrow (2 mm.) strip of cellulose tape was placed over the cotton plug to hold it in place. The cotton plugs were made as loose as sterility and efficiency would permit, and the cellulose tapes were made narrow to facilitate desiccation. Later work indicated the advisability of taping the cotton plugs after the lyophilization is accomplished. After preparation the vials were placed in a desiccator containing anhydrous $CaSO_4$. The desiccator was evacuated by means of a Cenco Hyvac pump, and the vacuum maintained until the cultures were ready to be sealed individually under vacuum.

After the cultures were lyophilized, the vials were placed in ⅝-inch Pyrex test tubes, which were then constricted in an oxygen flame. The constricted tube and contents were attached to a Cenco Hyvac pump and evacuated for 5 minutes before sealing off at the constriction. By using an oxygen flame to seal the Pyrex tube it was possible to make a thick-walled and durable tip. The cultures were graded on the basis of appearance as good, fair or poor. The "good" cultures were white and fluffy in texture. The "fair" were light in color and slightly puffed, but not fluffy. The "poor" were yellow and more resinous in appearance. The cultures were stored in the dark at room temperature.

Rehydration of Lyophilized Cultures

Inoculum was prepared in two ways from the lyophilized cultures. The first, and by far the more simple method, consisted of adding 1.5–2.0 ml. of sterile saline to the vial containing the dried culture. Stirring with a sterile loop hastened the rehydration. Reconstitution of the dried cells to 2 ml. restored them to their original concentration in the medium in which they were grown. This suspension of cells served as a convenient and satisfactory inoculum for 80–100 tubes when a hypodermic syringe and a number 24 needle was used to make the inoculations.

The lyophilized cultures may also be rehydrated in the vial with sterile saline or basal media and then transferred to a tube of medium for incubation before using as inoculum. This procedure offered no particular advantage when using *L. arabinosus*, aside from the fact that a greater volume of inoculum was obtained. When lyophilized *L. casei* cultures were transferred before being used as inoculum, acid production was slightly increased.

Media

The basal medium of Krehl, Strong and Elvehjem (4) was used in studying the response of the *L. arabinosus* cultures. The basal medium of Snell and Strong (5), modified to contain 2% glucose and 2% sodium acetate, was used in making the *L. casei* comparisons.

RESULTS

L. arabinosus Cultures

The *L. arabinosus* stock culture, from which the lyophilized cultures were prepared, was compared with a "good," a "fair," and a "poor"

culture after two weeks' storage. These cultures were transferred before being used as inoculum. Another "fair" culture was rehydrated but not transferred before use as inoculum. The 5 cultures gave an almost identical response, in terms of 72-hour 0.1 N acid production (Fig. 1). The values used in plotting the curves have had the blank titrations corrected to zero to facilitate comparisons.

Cultures were selected at random at two-month intervals throughout the year's storage study. At two months, four cultures were used; three
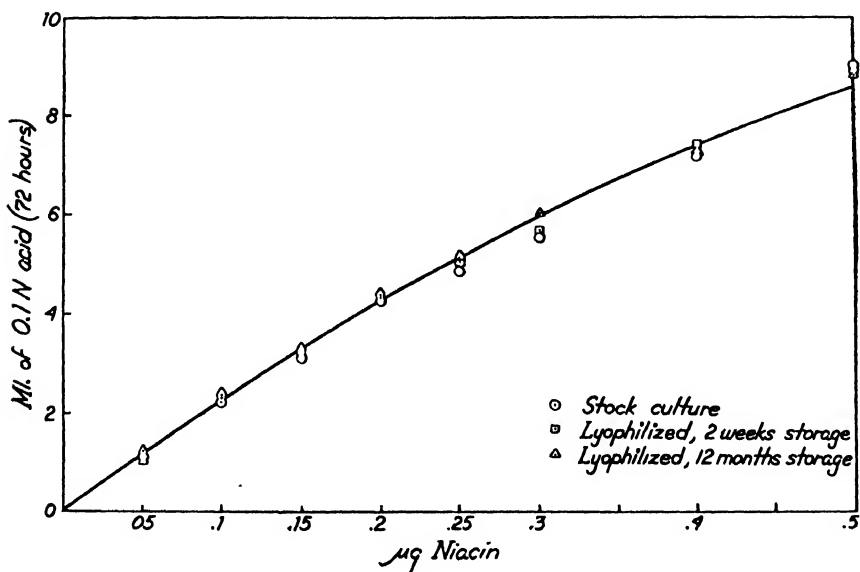
FIG. 1. The response to niacin of a stock *L. arabinosus* culture and lyophilized cultures after 2 weeks' and 12 months' storage.

were transferred, one was not transferred. At four months, one "good" culture was opened just before use as inoculum. At six months, one "good" and one "poor" culture were used without transferring. At eight months, a "good" culture was used, both before and after transfer, and a "poor" culture before being transferred. At ten months, a "good" and a "poor" culture were used immediately after rehydrating, and at twelve months, one "good," two "fair," and one "poor" culture were used as inoculum without transferring. The effect of storage of lyophilized *L. arabinosus* cultures is clearly shown in Fig. 1. The stock

culture is compared with cultures opened after two weeks' and twelve months' storage. The response of cultures opened at the other sampling periods has been omitted, since all were so similar in acid production and linearity.

In Fig. 2, the response of the five "poor" cultures shows a slightly lower acid production at the higher niacin levels than the other grades of cultures or the stock culture. At the lower levels of niacin this decrease in acid production is not evident.
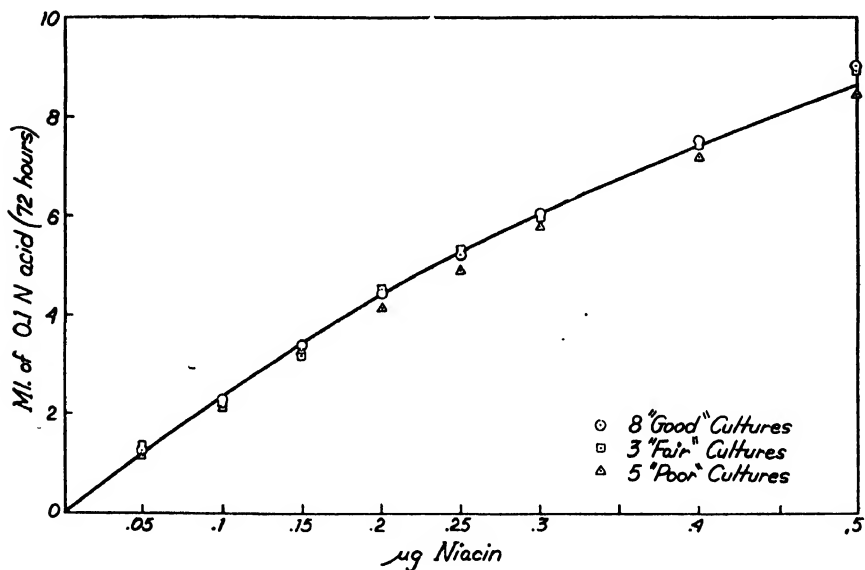
FIG. 2. Average response to niacin of lyophilized *L. arabinosus* cultures graded according to appearance.

L. casei Cultures

The *L. casei* stock culture from which the lyophilized cultures were prepared was compared·with a "good," a "fair," and a "poor" culture after one week's storage. These cultures were transferred before being used as inocula. Another culture graded as "fair" was rehydrated and used immediately as inoculum. The four lyophilized cultures gave a slightly lower acid production than the stock culture at all levels except 0.05 and 0.1 γ riboflavin (Fig. 3).

The cultures to be opened were selected at random at two-month intervals throughout the year's storage study. At two months, 7 cultures were opened; 3 were transferred, 4 were not transferred.

These cultures all showed a sharp decrease in acid production at all levels above 0.1 γ riboflavin (Fig. 3). This decline was evident for both the cultures which were transferred and those not transferred.

At the end of 4 months' storage, 5 cultures were tested. Three of the cultures were used both before and after transferring. At six, eight,
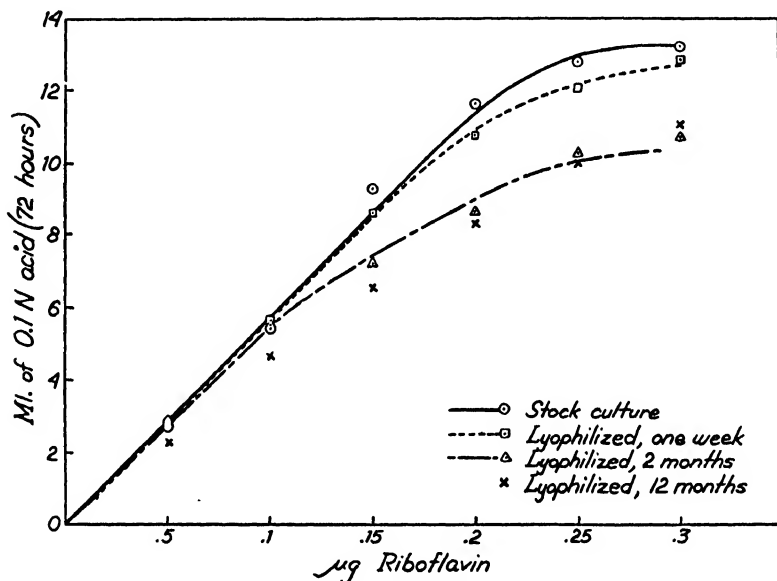
FIG. 3. Average response to riboflavin of a stock *L. casei* culture and lyophilized cultures after 1 week, 2 months', and 12 months' storage.

and ten months, representative cultures were rehydrated and used immediately as inocula. The cultures showed no change in response to riboflavin at these 4 sampling periods.

After 12 months' storage, 7 cultures were rehydrated and used as inocula. Transfers were made from 3 of the cultures. The poor-appearing cultures showed a more erratic and decreased acid production than did the better cultures. In Fig. 3, the comparison of the response of the *L. casei* cultures after storage indicates that the most serious decrease in acid-producing capacity occurs during the first two months of storage. After this first sharp decline, the cultures remain fairly con-

stant. However, the 12-month values indicate a slight decrease in acid production at the lower riboflavin levels.

In Fig. 4 is given the average acid production of all the "good," "fair," and "poor" cultures which were opened just before use as inoculum at the 2–12 month sampling periods. The cultures of the 3 different grades gave an almost identical response to riboflavin. Although the "poor" cultures showed a greater irregularity of response at the 12-month sampling period, the average acid production of all
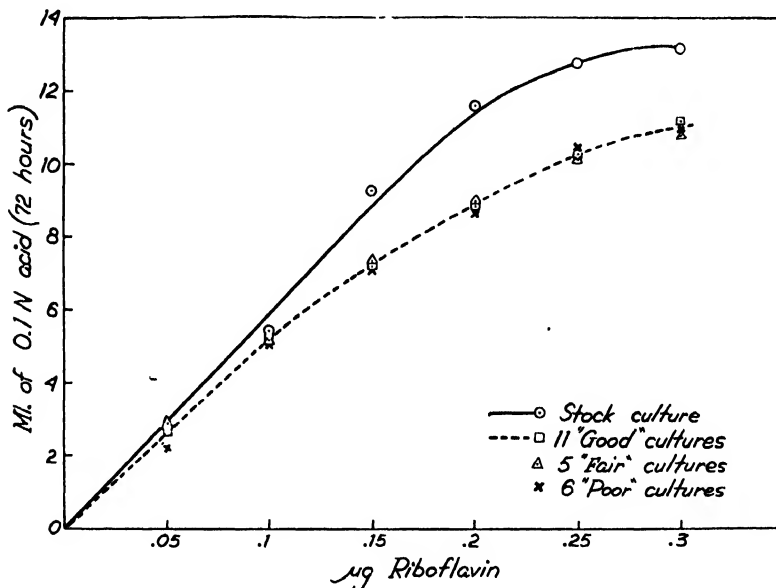
FIG. 4. Average response to riboflavin of the good, fair, and poor lyophilized *L. casei* cultures.

"poor" cultures opened during the year is similar to that of the "good" and "fair" cultures.

Seven cultures, including two "good," three "fair," and two "poor" samples, were used as inocula immediately after rehydrating and again after 24-hour growth in basal media with riboflavin added. Transfer of the cultures resulted in an increase in acid production especially at the higher levels of riboflavin (Fig. 5). The increase was not sufficient to restore the cultures to the original acid-producing capacity of the stock culture.

Results similar to those reported here have been obtained by Bathurst (6) who has communicated to us the following observations after a six-month storage of lyophilized cultures. *L. arabinosus* showed no change during storage. A low-acid-producing *L. casei* culture showed no change during storage. *L. fermenti* could be held satisfactorily in the lyophilized state, but to obtain normal growth in a 16-hour assay it was necessary to transfer the culture before using it as inoculum.
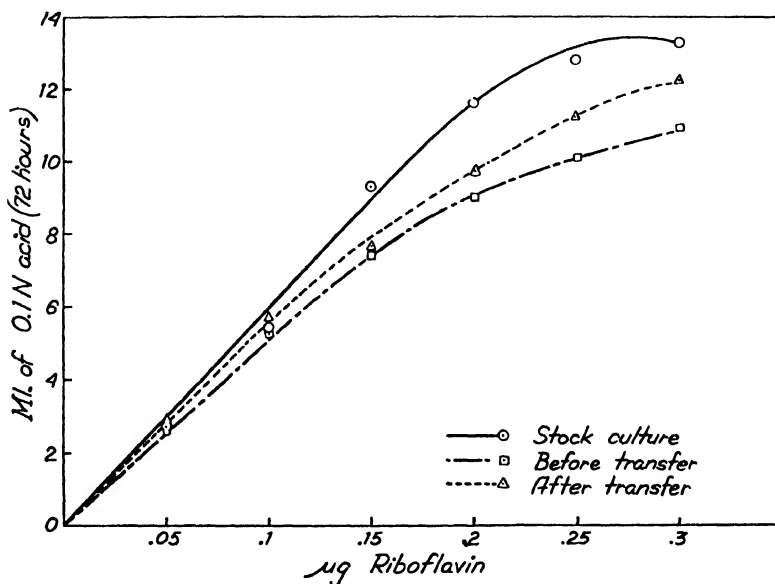
FIG. 5. Average response to riboflavin of seven lyophilized *L. casei* cultures when used as inocula before and after transferring through media.

SUMMARY

Following lyophilization, the *L. arabinosus* culture maintained its original acid-producing capacity over a 12-month storage period. The non-fluffy, resinous dehydrated cultures show a slight decrease in acid production at the higher niacin levels.

Acid production by all lyophilized *L. casei* cultures decreased during the first two months of storage, but remained constant thereafter until 12 months, when another slight decline in acid production occurred. Transfer of the *L. casei* cultures to media before using them as inocula increased the acid production. However, one transfer did not restore the cultures to their original acid-producing capacity.

These results indicate that the lyophile process is a simple, economical, and reliable means of maintaining bacterial cultures for microbiological assays.

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The Origin of Butyric Acid in the Fermentation of Threonine by *Clostridium propionicum*[1]

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INTRODUCTION

It is now well established that anaerobic bacteria generally form butyric acid by a condensation of two molecules of acetic acid or a derivative thereof. The evidence for this statement is of two types. When butyric acid-producing bacteria, such as *Clostridium aceto-butylicum* (1), *C. butylicum* (2), *C. kluyveri* (3, 4), *C. lacto-acetophilum* (5), and *Butyribacterium rettgeri* (6) are allowed to grow in a medium containing carbon-labeled acetate and a fermentable substrate, labeled butyric acid is formed. Secondly, two of the above mentioned organisms, *C. kluyveri* (7) and *C. lacto-acetophilum* (5), require acetate as a substrate and convert it more or less quantitatively to butyrate under suitable conditions.

Although acetate condensation is the most common mechanism of butyric acid synthesis, other mechanisms undoubtedly exist. Butyric acid is known to be formed by the oxidation of butanol by sulfate-reducing and methane-producing bacteria (8, 9), and there is also some evidence that butyric acid may be formed from higher fatty acids by organisms of the latter group (10). Recently, a fermentation of threonine by *Clostridium propionicum* was described (11) which produces butyric and propionic acids according to the equation

$$3CH_3CHOHCHNH_2COOH + H_2O$$
$$= CH_3CH_2CH_2COOH + 2CH_3CH_2COOH + 2CO_2 + 3NH_3.$$

It is unlikely that acetate is the precursor of butyrate in this process, because (a) acetate is not formed in detectable amounts from threonine, and (b) when acetate is formed by this organism from other substrates, such as alanine, no butyrate is formed.

To provide more direct evidence that acetate does not function as a precursor of butyrate in fermentations caused by *C. propionicum*, threonine was fermented in the presence of acetate labeled in both positions with C^{14}. The resulting mixture of butyric, propionic and acetic acids was separated by the chromatographic method of Elsden (12), and the radioactivity of the individual components was determined. Very little C^{14}, less than 0.5% of that added in the acetate, was found in the butyric acid fraction (Table I). The small amount of radioactivity in the butyric and propionic acid fractions can undoubtedly be attributed to an incomplete separation from the highly radioactive acetate.

The results of this experiment support the conclusion that acetate is not a precursor of butyrate. All the available evidence suggests that the C_4 chain remains intact during the conversion of threonine to butyrate. The process appears to be entirely analogous to the conversion of serine and other C_3 compounds to propionate. However, the individual steps of both processes are still unknown.

<center>EXPERIMENTAL</center>

The experiment was carried out with a washed cell suspension of *C. propionicum*. The organism was grown in the alanine medium described by Cardon and Barker (11), supplemented with 0.02% sodium carbonate. The cells from 1 liter of medium were suspended in 15 ml. of $M/20$, pH 7, phosphate buffer containing 0.02% $Na_2S \cdot 9H_2O$. To 10 ml. of this suspension were added 3 ml. of $M/10$ threonine, and 1.5 ml. of a solution containing 0.11 mM of doubly-labeled sodium acetate. The fermentation mixture was incubated *in vacuo* for 22 hours at 37°C. At the end of this period the quantity of ammonia formed was such as to indicate complete (98%) decomposition of the threonine.

<center>TABLE I</center>

<center>*Specific Activities of Fatty Acids Obtained by Fermenting Threonine in the Presence of Doubly-Labeled Acetate*</center>

Acid	Specific activity cts./min./mM
Acetic	139×10^3
Propionic	1.3×10^3
Butyric	0.7×10^3

The volatile acids were separated from the fermentation mixture by steam distillation, concentrated to a small volume as the sodium salts, acidified with sulfuric acid and extracted with ether in a micro Soxhlet apparatus. The ether was distilled off and the acids were dissolved in 5 ml. of chloroform. The acids were then separated by partition chromatography (12). The 3 bands were collected separately and the corresponding acids were identified by Duclaux distillation as butyric, propionic, and acetic acids, respectively. The acids were then titrated, concentrated to a small volume, and the sodium salts were spread on aluminum disks for radioactivity measurements by means of a Geiger-Müller counter (13).

The specific activities of the salts are given in Table I. The radioactivity of the propionate and butyrate samples was negligible in relation to that of the acetate, and was evidently due to a slight contamination with acetate. The propionate was more heavily contaminated than the butyrate because it was more closely associated with the acetate on the partition chromatogram.

SUMMARY

By the use of C^{14} it was shown that acetate is not an intermediate in the formation of butyric acid from threonine by *Clostridium propionicum*.

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Studies on Glycolysis of Brain Preparations. IV [1,2]

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INTRODUCTION

A great bulk of literature on the measurement of glycolytic rates for free sugars, phosphate esters of sugars, or other intermediaries of the glycolytic cycle has accumulated during the last 10 years. The measurements were made on tissue preparations (homogenates, extracts or suspensions of dried powder) from various organs and animals. From our preceding papers (1, 2, 3) it is obvious that it would be utterly futile to draw any conclusions about the activity in the living cell from the different enzymatic rates encountered for different free sugars, hexosemonophosphates, and hexosediphosphate, unless numerous factors are carefully checked and considered.

Most of the conflicting results of the authors working in this field can be explained (if the influence of cell permeability is excluded) by: (1) variations of stability of the individual enzymes, varying from one organ and animal to the other, and depending on the applied procedures; (2) activity of the autolyzing enzymes, especially of those which destroy coenzymes; and (3) disturbance of the normal interplay of the stepwise reactions by lack of coordination of their speed. As will be shown in a paper which is to follow, hexosemonophosphate is glycolyzed much more quickly in extracts of sarcoma of rats and mice than in brain extract, but glucose much more slowly. The only conclusion to draw from such an observation (together with other concomitant facts) is that the hexokinase from sarcoma is damaged much more than the phosphohexokinase. This is not the case in brain. If pyruvate

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increases the rate, especially in extracts from rat sarcoma but not in extracts from brain, it shows that an enzyme leading to pyruvic acid is more damaged than the enzymes of the oxidation-reduction step. This enzyme, incidentally, is the enolase which is even more sensitive in rat sarcoma than in mouse sarcoma.

The speed of the full glycolytic cycle obtained in homogenates and extracts depends on the coordinated function of a score of partial enzymes and a suitable checking of interfering enzymes. Only by considering the changes which occur simultaneously in the different components of the system can a consistent picture be obtained and deductions applicable to the living units be drawn from the dispersed systems.

To demonstrate this point more clearly, we publish here further experiments dealing with the turnover of various hexoses and hexose esters, and with some inhibitors of glycolysis in the homogenates and extracts from brain tissue, and also in extracts from acetone powder of brain.

METHODS

The methods, in general, were the same as those used in the preceding papers. Lactic acid was determined either manometrically or chemically. For the chemical determination, the apparatus of Lieb and Zacherl was used (4). The chemical method was used when additions to the glycolyzing mixture had to be made at frequent intervals. When it was desired to add ATP[3] to the homogenate continuously during glycolysis, we let the ATP solution drip in from a fine capillary pipette connected to a section of rubber tubing. The flow was regulated with a Hoffmann screw clamp; the period of addition was 20–30 minutes, and the solution was stirred occasionally.

Acetone powder of brain was obtained by rubbing ice-cold rabbit brain with a small amount of acetone and pouring the pulp into 10 times its volume of ice-cooled acetone. The powder was washed with acetone, dried *in vacuo* over sulfuric acid, and stored in the ice box. This preparation was similar to those from muscle or other tissues and also to that which Cori and coworkers have recently used for their studies on hexokinase (5). An extract of the powder with about 16 volumes of watery solution was used as enzyme. The solution used most frequently was a "modified" Ringer, containing 2–3 parts of an isotonic mixture of NaCl (10 cc. of 0.9%), KCl (0.5 cc. of 1.15%), and $MgSO_4$ (0.8 cc. 3.82% $MgSO_4 \cdot 7H_2O$), and one part of $NaHCO_3$ (1.3%). For glycolysis experiments the Ringer solution contained 3% of nicotinamide.

In contrast to our former routine procedure we prepared the extract without preliminary freezing of the homogenate in dry ice; we centrifuged the extract for 5–6

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[3] Abbreviations used: ATP = adenosine triphosphate; ADP = adenosinediphosphate; ATPase = enzyme splitting first labile P-group of ATP; apyrase = adenylpyrophosphatase, enzyme splitting both labile P groups; pyro-P = labile P of ATP and ADP; HDP = hexosediphosphate; HMP = hexose monophosphate.

min. at 4500 r.p.m. and used 0.3 cc. of extract from an homogenate made with 3.6 parts of modified Ringer solution in *ca.* 0.9 cc. total vol. With the former higher dilution of the extract and shorter centrifuging, as well as with repeated freezing and thawing of the homogenate, one obtains Q_{La} of 40–60 with glucose, but also greater variations and occasional failures. With the present procedure the Q_{La} are smaller, about 30, but more consistent.[4]

HDP formed during glycolysis was determined by means of the zymohexase reaction in the presence of KCN (6); neutralized trichloroacetic acid filtrate of the glycolytic mixture was incubated with the zymohexase fraction of muscle extract or with dialyzed extract of acetone powder from muscle. In the presence of $M/10$ KCN, neutralized in borate buffer, all HDP is transformed into the cyanhydrin of triosephosphate, determined as alkali-saponifiable P.

1. Differences between Glucose and Fructose

In two preceding papers (1, 2) the reason for the difference in the glycolytic rates of glucose and fructose was discussed. It was shown in a preliminary note (2) that the difference formerly demonstrated in brain slices (7), or homogenates (1), can be observed in extracts when a suitable very low range of ATP and HDP concentrations is used. This range is only slightly above the limit necessary to start a continuous glycolysis. If the concentration is very little higher, the difference between the two sugars disappears.

Moreover, the difference is also dependent upon a special dilution of the extract and, for the range used here, dependent on adherence to our experimental conditions. We therefore give several more experiments in Table I and Figs. 1 and 2. For our conditions 2–10 γ pyro-P of ATP/cc. and 7–15 γ P of HDP were best suited, but with more than 8 γ pyro-P, less than 12 γ P of HDP should be used.

As may be seen from Table I the relative rates are somewhat variable, and sometimes fructose turnover stops completely after a short interval. In Fig. 1 the rates of glycolysis are given after different times of centrifugation of the extract. This answers the possible objection that after only 5 minutes of centrifugation at 4500 r.p.m. some particles may still remain suspended, and might contain adsorbed hexokinase. Actually, with great dilution of the homogenate the influence of particles becomes negligible as was shown in the first paper. Fig. 1 shows that, after 10 minutes of centrifugation, where the extract is completely

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[4] Centrifuging the same homogenate for 6 min. at 2800 instead of 4500 r.p.m. increases the Q_{La} value to 40–55, but the results are somewhat less reproducible, probably because of the higher content of apyrase.

TABLE I

Glycolysis of Glucose and Fructose in Extract with Low ATP

No.	Time	Total volume	Sugar added g =glucose f =fructose	Sugar	ATP γ pyro-P	HDP γ P	Q_{La} Mano-metric	Q_{La} Chem-ical
	mins.	*cc.*		*mg.*				
277	40	1.15	—	—	15.0	17	2.4	0.5
	40	1.15	g	4	15.0	17	24.6	22.6
	40	1.15	f	4	15.0	17	18.8	17.5
	40	1.15	g	4	5.5	17	13.4	13.7
	40	1.15	f	4	5.5	17	5.3	1.5
281	60	1.20	—	—	5.0	14	0.0	
	60	1.20	g	3	5.0	14	22.0	
	60	1.20	f	3	5.0	14	11.5	
	60	1.20	g^a	3	5.0	14	14.0	
	60	1.20	f^a	3	5.0	14	5.3	
283	60	0.95	—	—	5.0	7	0.0	
	60	0.95	g	2	5.0	7	25.0	
	60	0.95	f	2	5.0	7	6.2	
	60	0.95	g	2	2.5	7	17.5	
	60	0.95	f	2	2.5	7	1.4	
	60	0.95	g	2	1.3	7	5.6	
	60	0.95	f	2	1.3	7	1.2	
284	60	0.95	—	—	5.0	7	0.5	
	60	0.95	g	2	5.0	7	19.0	
	60	0.95	f	2	5.0	7	2.8	
	60	0.95	g	2	55.0^b	7	28.6	
	60	0.95	f	2	55.0^b	7	27.0	
289	60	0.95	—	—	6.2	7	0.5	
	60	0.95	g	2	6.2	7	3.9	
	60	0.95	f	2 .	6.2	7	1.5	
	60	0.95	g	2	6.2	11.5	13.1	
	60	0.95	f	2	6.2	11.5	1.5	
	60	0.95	g	2	10.5	11.5	20.0	
	60	0.95	f	2	10.5	11.5	2.5	
	60	0.59	g	2	55.0^b	11.5	26.0	

^{a} Centrifuged 10 min. (4500 r.p.m.) instead of 5 min.
^{b} Controls with optimal ATP.

clear, the same difference exists as after 5 minutes, although all rates are lowered. The difference, therefore, can be fully accounted for by the low ATP (in conjunction with the low HDP) without having re-
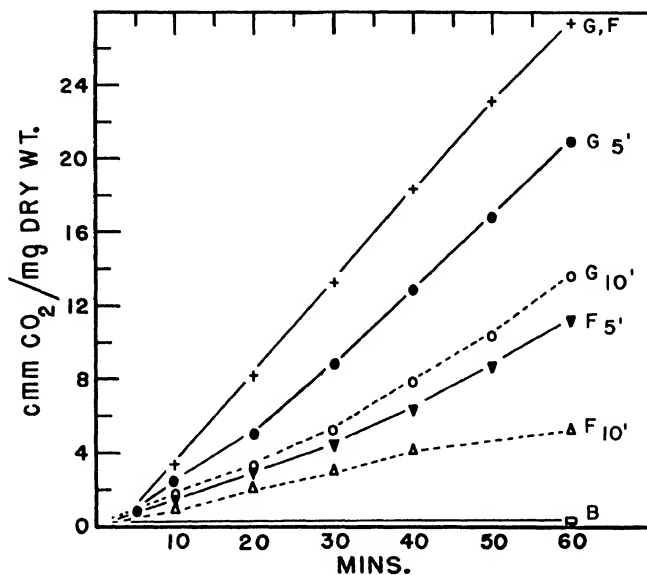
Fig. 1. Glycolysis of glucose and fructose in centrifuged extract with different times of centrifugation (4500 r.p.m.) (No. 281). +G, F = glucose and fructose with optimal amount of ATP (50 γ pyro-P/cc.). ●G$_{5'}$ and ▼F$_{5'}$ = glucose and fructose with 5 min. centrifuged extract, 5 γ pyro-P/cc. ○G$_{10'}$ and △F$_{10'}$ = glucose and fructose with 10 min. centrifuged extract, 5 γ pyro-P/cc. □B = Blank. Ordinate: mg. dry weight, referring to the tissue from which the extract is made, not to the actual dry weight in the extract.

course to a difference between adsorbed and dissolved hexokinase. Fig. 2 gives examples of the variation of the amounts of ATP in the extract. On the other hand, the regular difference between glucose and fructose as found in the homogenate could be abolished either by adding ATP anew at very short intervals (1), or by regenerating it continuously by phosphocreatine (3). A third method consists in keeping HDP as well as free sugar on a high level. This is shown in Fig. 3 compared with the effect of the single components. Fructose alone gives practically the same as the blank, but fructose plus HDP gives considerably more than
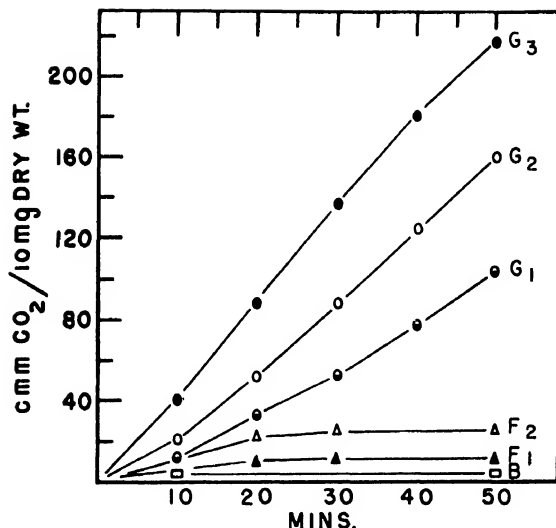
Fig. 2. Glycolysis of glucose and fructose (G and F) in extract with different amounts of ATP (No. 289) 11.5 γ HDP in all samples.

◖G_1 = 6.2 γ pyro-P	▲F_1 = 6.2 γ pyro-P
○G_2 = 10.5 γ pyro-P	△F_2 = 10.5 γ pyro-P
●G_3 = 55 γ pyro-P	□B = Blank

the sum of both singly. The main difference, therefore, between homogenate and extract, in regard to glycolysis, consists in the high concentration of "apyrase" bound to the structural elements and removed by centrifugation. This lowers the concentration of ATP in the homogenate to a range where the different affinity of glucose and fructose becomes apparent, unless ATP is more rapidly regenerated than by the glycolytic cycle alone. Only one feature cannot be duplicated in the extract. In homogenate and slices higher concentrations of fructose give, fairly regularly, a higher turnover rate, intermediate between low fructose and glucose. In extracts, the turnover rate of fructose is not affected by the concentration in the range where the difference of glucose and fructose becomes visible. Here adsorption may play a role.

No difference was found when ADP was used instead of ATP. Indeed, transphosphorylation was exactly the same (Table II) with ADP and ATP in the reactions:

Hexose + ADP → hexosemonophosphate + AA,
Hexose + ATP → hexosemonophosphate + ADP.
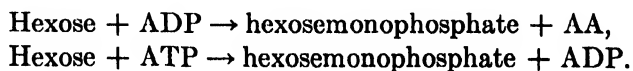
From Table I it follows that, with 2–6 γ pyro-P/cc., the turnover rate of glucose and fructose in extracts is about that of the living tissue from which the extract is prepared. But analytically, 180 γ pyro-P of ATP are found in 1 g. of brain tissue (8). This ATP, of course, is engaged in many other reactions besides those of the glycolytic cycle. Moreover, it is a fair assumption that most of this ATP is not freely accessible to the glycolytic enzymes but separated from them by cell structures. Without laying too much stress on the quantitative side, it may be presumed that only 2–5% of the total amount is in free
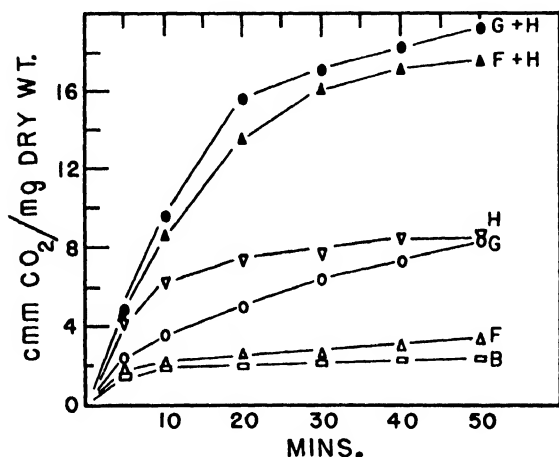
FIG. 3. Glycolysis in complete homogenate with glucose and fructose, with and without HDP addition (No. 275). 0.3 cc. homogenate (15 mg. dry weight) in 1.2 cc. total vol. in each sample. ▽H = HDP with 450 γ hexose (112 mm.³ CO₂), which can give *in maximo* 7.5 mm.³/mg. dry weight. ○G = Glucose (4 mg.), △F = Fructose (4 mg.), □B = Blank, ●G + H and ▲F + H = mixtures of 4 mg. glucose or fructose with 450 γ hexose of HDP.

equilibrium with the glycolytic enzyme system; this would correspond to the concentration of ATP in the extract under the above-discussed conditions.

As a corollary, the rate of glycolysis in the complete homogenate was measured with continuous addition of ATP, compared with only one addition, and also with the turnover in the extract made from the same homogenate, after one addition. A similar experiment has already been published in our first paper (1) (Table VII, p. 422), where 50 γ pyro-P of ATP were added to the homogenate every two minutes. In

TABLE II

Transphosphorylation with ATP and ADP

No.	Time	Sugar	Pyro-P added as	Pyro-P present γ	Increase of inorganic P γ	7-min. P γ	7-min. P Corrected for blank γ
	mins.						
290	0	—	ATP	171	—	—	—
	10	—	ATP	163	15.6	−7.7	—
	10	Glucose	ATP	132	8.7	−29.8	−37.5
	10	Fructose	ATP	140	8.2	−22.0	−29.7
	0	—	ADP	174	—	—	. . .
	10	—	ADP	157	15.6	2.4	. .
	10	Glucose	ADP	124	12.8	−38.0	−35.6
	10	Fructose	ADP	126	16.5	−32.2	−29.8

this way Q_{L_a} values were obtained for glucose: 21.8 and 20.3, and for fructose: 15.4 and 21.2, while with one addition the value for glucose was 9.3 and for fructose, 3.2.

Actually, no further increase of lactic acid formation was obtained with continuous dropping in of ATP compared with additions every two minutes. This seems, therefore, to give the optimal ATP concentration in the homogenate. Here about 900 γ pyro-P are added to 0.5 cc. homogenate (approx. 20 mg. dry weight) in half an hour. Except for one experiment which is not reproduced because some irregularity probably occurred, lactic acid formation was the same with continuous or two minute interval addition to the homogenate, on the one hand, and with one addition to the extract (Table III). With a single addition of 30–60 γ pyro-P to the homogenate, on the other hand, low values are obtained. If the blank values are subtracted, only a trace of lactic acid is formed with fructose. In No. 382, a further experiment with two minute additions was made. Two samples of homogenate were incubated immediately; the others, and likewise the extract, were left 20 minutes at room temperature and 15 minutes at 38°C. to approach the conditions of the manometric experiments. In this latter case the lactic acid formation was even somewhat greater.

The calculated Q_{L_a} values (approximately 25 over the blank) correspond to those obtained manometrically under similar conditions. There remains a trifling difference between glucose and fructose in the homogenate, not visible in the extract. This may result from incompletely uniform dispersion of the particles, which may still include some hexokinase not accessible to the added ATP. A slightly different metabolic speed for glucose and fructose would then obtain.

These experiments, on the whole, give a conclusive proof that the

TABLE III

Formation of Lactic Acid in Homogenate and Extract with Single or Continuous Addition of ATP

No.	Time	H = Hom. E = Ext.	Sugar gl=gluc. fr=fruct.	mg.	Initial vol.	Pyro-P of ATP continuous addition γ	Pyro-P of ATP one addition γ	Lactic acid ATP cont. added γ	Lactic acid ATP added once γ	Q_{La} calc. ATP cont. added	Q_{La} calc. ATP added once
373	min. 23	H	—	—	1.2	1270	30	73	73	3.0	3.0
		H	gl	4	1.2	1270	30	525	157	21.6	6.6
		H	fr	4	1.2	1270	30	475	97	19.2	4.0
		E	—	—	1.2		30		73		3.0
		E	gl	4	1.2		30		435		18.0
		E	fr	4	1.2		30		447		18.4
374	30	H	—	—	2.0		60		20		0.4
		H	gl	4	2.0	1300	60	638	141	13.1	2.9
		H	fr	4	2.0	1300	60	526	35	10.8	0.74
		E	—	—	2.0		60		80		1.65
		E	gl	4	2.0		60		586		12.1
		E	fr	4	2.0		60		572		11.8
383	30	H	—	—	2.2		60		218		5.4
		H	gl	8	2.2	960*		1025		25.7	
		H	fr	8	2.2	960*		1020		25.5	
		H**	gl	8	2.2	960*		1266		31.7	
		H**	fr	8	2.2	960*		1163		29.1	
		E**	—	—	2.2		60		10		0.25
		E**	gl	8	2.2		60		1135		28.4
		E**	fr	8	2.2		60		1135		28.4

* 63 γ pyro-P added every two minutes.
** Homogenate and extract after 35 minutes standing.

difference between the two sugars in the homogenate is due to low concentration of ATP because of the excess of apyrase.

2. Acetone Powder Extract

If a watery extract of acetone powder of brain is made as described above, glycolysis is somewhat altered. Two main changes occur. The apyrase is, in great part, destroyed by the acetone treatment, as was

observed earlier with muscle extract and other tissue extracts, and the hexokinase is damaged more than the phosphohexokinase. This can be seen by the transphorsphorylation with ATP in presence of NaF, but still more conspicuously in the total glycolytic turnover.

Only the experiments with hexosemonophosphate will be described here. If no apyrase were present, then for every mol of HMP which forms lactic acid, three mol HDP should accumulate instead of one mol provided free sugar is glycolyzed or fermented (equation of Harden and Young). This follows from the fact that 4 energy-rich posphate bonds are generated by the turnover of one hexose unit. With free sugar, two are needed to phosphorylate the next hexose unit which
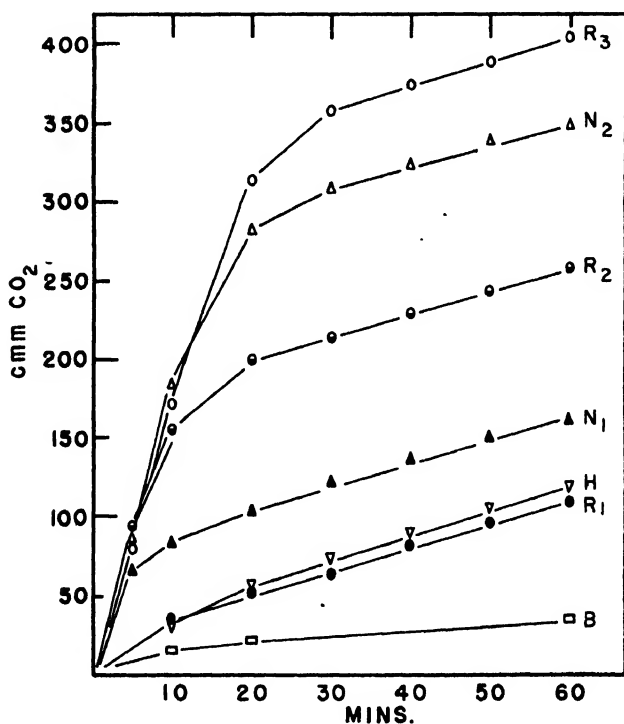
FIG. 4. Glycolysis of HMP in extract of acetone powder of brain. R = Robison ester; N = Neuberg ester (No. 371). Different amounts of esters containing the following amounts of hexose: ●R_1, ◐R_2, ○R_3 = Robison ester with 1.5, 3.0., and 5.0 mg. hexose. ▲N_1 and △N_2 = Neuberg ester with 2.2 and 5.6 mg. hexose. ▽H = HDP with 2.35 mg. hexose. In each sample 30 mg. dry powder extract in 1.65 cc. total vol. □B = blank.

ferments while two remain in excess and form one mol HDP. But if HMP glycolyzes, only one is needed to form the mol of HDP which ferments, and three remain in excess and can phosphorylate three mol HMP to HDP. Such a behavior can be observed in our case. Because the apyrase is not totally absent, only about 2.5 mol HDP accumulate for one mol HMP glycolyzed. The monoester reacts at very high speed until about a quarter of the total is split to lactic acid. Then the rate drops exactly to that of HDP. This can be seen in Fig. 4. The amount of HDP formed from HMP was measured at the end by zymohexase in the presence of KCN (5, 6).

The total balance sheet of experiment No. 371, shown in Fig. 4, is given in Table IV. No difference exists between Neuberg ester (pure

TABLE IV

Turnover of Hexosemonophosphate in Extract of Acetone Powder
1 hour 38°C. HDP measured by zymohexase; lactic acid, manometric

Ester N = Neuberg R = Robison	Hexosephosphate content at start		Lactic acid formed		Inorg. P disapp. γ	HDP found by zymohex.		Hexose in total accumulated HDP[a]	Ratio[b]
	mg. hexose	org.P	rapid period	total		γ P	mg. hexose		
			mg.	*mg.*				*mg.*	
R	1.5	260	0.15	0.45	−148	210	0.6	0.9	2.5
R	3.0	520	0.8	1.04	−278	576	1.8	2.04	2.5
R	5.0	870	1.45	1.60	−531	1130	3.4	3.55	2.5
N	2.2	·380	0.4	0.64	−139	180	0.55	0.70	1.8
N	5.6	950	1.2	1.44	−260	765	2.3	2.55	2.1
HDP	2.35	780		0.5	612[c]	532	1.55		

[a] This includes the amount of HDP split in the slow period after all HMP has disappeared.

[b] Ratio: $\dfrac{\text{Mol. HDP accumulated in total}}{\text{Mol. HMP split in rapid period}}$.

[c] Inorganic P in beginning.

fructose monophosphate) and Robison ester (containing 70% glucose monophosphate and 30% fructose monophospate.) Somewhat more than double the amount of HMP accumulates as inorganic P disappears. A second experiment gave the same results.

3. INHIBITORS OF GLYCOLYSIS

What intermediary reactions are attacked is still unknown for most inhibitors of glycolysis. Only for NaF and iodoacetic acid are

these reactions clearly established. The inhibition by adrenochrome, first described by Randall (9), is investigated jointly with this author in the following paper.

Phlorhizin was found by E. Lundsgaard to inhibit glucose phosphorylation in tissue extracts (10). He assumed this inhibition to be responsible for "phlorhizin diabetes" or, more strictly, for the disturbed reabsorption of glucose from the tubuli into the venous blood. The inhibition of phosphorylation becomes conspicuous in a 0.01 M solution. According to Shapiro (11) the effect of phosphorylation is indirect, the most sensitive reaction being the oxidation of pyruvate or citrate. Because these reactions are inhibited by 0.001 M phlorhizin the rephosphorylation of ATP is blocked, and in this way the phosphorylation of glucose is inhibited.

We find the anaerobic glycolysis in brain and tumor slices and in homogenates less sensitive. Here 8×10^{-3} M inhibits about 60%. The inhibition in extracts and homogenates is equal for HDP and free sugar, in contrast to the inhibition by adrenochrome. This shows that not the hexokinase but an enzyme effective after the phosphorylation of sugar is being inhibited. The decisive step is the dephosphorylation of phosphopyruvate by ADP.

If relatively large amounts of phosphoglyceric or phosphopyruvic acid are incubated, together with small amounts of ATP, in the presence of homogenate, or extracts, the rate of dephosphorylation depends on the reaction:

$$\text{phosphopyruvate} + \text{ADP} \rightarrow \text{pyruvate} + \text{ATP}.$$

That this reaction is specifically inhibited by phlorhizin can be demonstrated by addition of NaF. Apyrase is partially inhibited thereby, but the relative rates of dephosphorylation of pyruvate, with and without phlorhizin, are the same as in absence of NaF (Table V).[5]

o-Phenanthroline, which inhibits fermentation and glycolysis (12), probably by complex formation with heavy metal, gives about 90% inhibition of glycolysis in brain extract in concentrations of 8×10^{-3} M; a 3×10^{-3} solution gives about 50% inhibition. HDP turnover is inhibited a little less. Because o-phenanthroline interferes with the phosphate determination, the point of attack could not be ascertained. $ZnSO_4$

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[5] The sarcomas used in some of the experiments of Tables V and VI were transplanted rat sarcoma 303, induced by methylcholanthrene by Dr. Margaret Lewis of the Wistar Institute, to whom we are obliged for this help.

TABLE V

Inhibition of Transphosphorylation by Phlorhizin
1.3 cc. total vol.; 17 γ pyro-P of ATP

No.	Prep.	Time	Phlorhizin M	P donor[a]	Increase in inorg. P γ	Inhibition
		mins.				*per cent*
322	Brain homog.	10	—	pp	69	
	Brain homog.	10	0.01	pp	28	60
	Brain ext.	10	—	pg	94	
	Brain ext.	10	0.01	pg	41.5	56
326	Brain ext.	10	—	pp	78	
	Brain ext.	10	0.01	pp	30.8	60
	Brain ext.	10	—	pg	99	
	Brain ext.	10	0.01	pg	42	58
	Brain ext.[b]	10	—	pp	18.8	
	Brain ext.[b]	10	0.01	pp	7.3	56
323	Sarcoma ext.	15	—	pp	92	
	Sarcoma ext.	15	0.01	pp	50.6	45
	Sarcoma ext.	15	—	pg	71.4	
	Sarcoma ext.	15	0.01	pg	52.2	30
324	Sarcoma ext.	10	—	pp	76	
	Sarcoma ext.	10	0.01	pp	40	48

[a] pp = phosphopyruvate; pg = 3-phosphoglycerate.
[b] 0.07 N NaF added.

and FeSO₄ in small amounts did not abolish the inhibition, as was found by Warburg and Christian (13) for zymohexase of yeast. Only amounts of FeSO₄ sufficient to remove the total o-phenanthroline as complex salt relieved the inhibition.

Among the pyrimidine derivatives alloxan was tested. It inhibits glycolysis in extract 90% in 1.5×10^{-3} M and 50% in 8×10^{-4} M (see also Gemmill, 19).

4. Narcotic Inhibition

To get a closer picture of the physicochemical state of the enzymes in our preparations, we tested the narcotic inhibition by some higher members of homologous series, especially phenylurethane and *n*-octyl- and capryl alcohols in saturated solution. According to O. Warburg these substances completely inhibit respiration, assimilation, and fermentation in living cells and granules, but do not inhibit fermentation in extracts (14). This difference is linked to the adsorption of the

TABLE VI

Narcotic Inhibition of Glycolysis in Preparations of Brain and Sarcoma

No.	Type of preparation	Time	Substrate	Narcotic (satur.)[a]	mm.³ CO_2 formed		Inhibition
					in control	with narcotic	
		nims.					*Per cent*
377	Brain homog.	40	HDP	Capryl.	223	250	0
				n-octyl.		216	0
	Brain extract	60	gluc.	n-octyl.	344	277	20
387	Brain extract	40	gluc.	n-octyl.	336	281	17
				phenyl.		34	90
	Brain extract	40	HMP	phenyl.	327	199	40
382	Brain extract	60	gluc.	n-octyl.	290	230	20
				phenyl.		34	90
	Brain extract	30	HMP	n-octyl.	288	247	14
				phenyl.		165	43
384	Brain extract	60	HDP	phenyl.	195	88	50
	Brain extract	60	gluc.	n-octyl.	373	268	28
387	Brain extract	60	gluc.	phenyl.	315	49	85
				phenyl.[b]		100	68
380	Sarcoma slice[c]	40	gluc.	n-octyl.	24	0.8	97
				phenyl.		8.4	65
	Sarcoma extract	40	HMP	n-octyl.	283	129	55
	Sarcoma extract	40	HDP	n-octyl.	258	146	45
386	Sarcoma extract	30	HMP	capryl.	325	230	33
				phenyl.		190	42
	Sarcoma extract	30	HDP	capryl.	225	90	60
				phenyl.		140	38

[a] Narcotics: capryl. = capryl alcohol
       n-octyl. = n-octyl alcohol
       phenyl. = phenylurethane.

[b] Phenylurethane solution half-saturated at 38°C.

[c] mm.³ CO_2/mg. dry weight/hr. = Q_{La}.

enzymes on the living interfaces. Owing to the surface activities of the narcotics, their concentration on these places would be much higher than in solution.

Because the apyrase is strongly adsorbed on the cell structures and cannot be removed by washing with Ringer solution, we expected that it would be inhibited by octyl alcohol. But this was not the case. Neither in the complete homogenate nor in the particles washed twice with Ringer solution could any inhibition of the apyrase be detected. Accordingly, the turnover of HDP in the complete homogenate, which depends on the speed of the apyrase reaction, is not influenced by saturated octyl alcohol (Table VI, No. 377).

On the other hand, octyl alcohol gives an inhibition of 20–25% of glycolysis in brain extract with glucose and HMP, and 40–50% inhibition of the turnover of HMP in sarcoma extract.

Still more conspicuous is the effect of saturated phenylurethane; glycolysis of glucose in the brain extract is often nearly 90% inhibited, while that of HMP is about 40% inhibited.

This seems to show that the hexokinase of brain, although in apparent solution, behaves towards higher members of the narcotic series in a manner intermediate between a true dissolved enzyme and an adsorbed one, while the adsorbed apyrase behaves like a dissolved enzyme. Probably only some adsorbed enzymes, and not all, are responsible for the degree of inhibition displayed by the living cell toward the higher narcotic members.

DISCUSSION

The importance of hexokinase for the turnover rate of sugars, as emphasized by the recent work of Cori and his school (5), again becomes conspicuous in the foregoing experiments. It cannot be definitely stated yet whether the differences between glucose and fructose are due to the presence of two different hexokinases, glucokinase and fructokinase, as they were called by G. T. Cori and M. W. Slein (15). It remains also for further investigation to find out whether the first phosphorylation product is fructose-6-phosphate or fructose-1-phosphate. Because both would yield, on further phosphorylation, fructose-1,6-diphosphate, the course of glycolysis would be the same in both cases. In unpublished experiments O. Meyerhof and H. Green found that, during phosphatatic synthesis, only 20% fructose-6-phosphate, about

75% fructose-1-phosphate, and some HDP formed from fructose and phosphate. On the other hand, the findings of Gottschalk (16), that yeast ferments only fructofuranose but not fructopyranose, because here the 6-position is blocked by the oxygen bridge, leads to the conclusion that, in yeast, fructose-6-phosphate is the phosphorylation product of fructose. However, in this case, the uniformity of crystallized hexokinase (17, 18) seems to show that no separate fructokinase exists in yeast. The conditions of our experiments make it impossible that the differences encountered between glucose and fructose at low ATP concentration could be ascribed to the inaccessibility of fructopyranose to phosphate.

Summary and Conclusions

1. The fact described in a preliminary note (2) is demonstrated by further experiments, *viz.*, the difference in the turnover rate of glucose and fructose as found in slices and homogenates of brain can also be obtained in the centrifuged extract, when the concentrations of ATP and HDP are lowered to the limit where a continuous glycolysis is just possible. On the other hand, the difference in the homogenate can be abolished by continuous addition, or by repeated addition at short intervals, of ATP (about 1000 γ pyro-P/30 minutes) which compensate for the rapid loss of ATP brought about by the structurally bound apyrase. In this case Q_{La} values practically identical with those in the extract with *one* addition of 30–60 γ pyro-P of ATP are obtained in the homogenate. While, in the homogenate, the ATP is kept low by the adsorbed apyrase, one must assume that in the living cell the acting concentration is similarly low by separation of the bulk of ATP from the active enzymes by means of cell structures and inert proteins.

2. Acetone powder extract of brain, in which most of the apyrase is destroyed, is still able to metabolize HMP, but only one-fourth of the total with high speed, while the rest is transformed to HDP. This is the direct consequence of the stoichiometric coupling, in which four energy-rich P bonds are generated for each hexose unit which ferments. With free sugar *one* unit HDP accumulates for one mol sugar broken down; with HMP three mol are expected to accumulate for one mol HMP broken down. This is approximately the case.

3. Some inhibitors of glycolysis were tested in our system. Phlorhizin attacks preferentially the transphosphorylation between phosphopyru-

vate and ATP. Octyl alcohol and phenylurethane, higher members of the homologous narcotic series, inhibit hexokinase and phosphohexokinase to various extents. Glucose turnover in brain extract is inhibited 50–90% by saturated phenylurethane, and HMP turnover about 40%. Octyl alcohol inhibits the same reactions 20–30%. On the other hand, adsorbed apyrase is not inhibited by saturated octyl alcohol. Hexokinase in solution behaves, therefore, more like the structurally bound cell metabolism than does the adsorbed apyrase.

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The Inhibitory Effects of Adrenochrome on Cell Metabolism[1]

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1. STUDIES ON BRAIN PREPARATIONS

After Randall had shown (1) that, under special conditions, glycolysis of brain homogenate was inhibited more than 50% by about $4 \times 10^{-6} M$ adrenochrome (0.7γ/cc.), it seemed worthwhile to find the locus of attack of this inhibitor.

We have ascertained that an inhibition similar to that described by Randall can be obtained in the system described in the preceding papers (2-5). Here a centrifuged extract of brain homogenate is used, made with Mg-containing "modified Ringer." Glucose (or fructose or hexosemonophosphate) serves as glycolyzing substrate. Only a slight trace of HDP[2] (approximately 5γ P/cc.) is used as a primer instead of 150γ P of HDP.

The adrenochrome was prepared by Dr. R. Duschinsky and some o-naphthoquinones by Dr. Aeschlimann in the Scientific Department of Hoffmann-La Roche, Inc. The additions to the extracts (ATP, cozymase, *etc.*) were the same as in the preceding papers. Only glutathione was omitted, as this substance reacts with adrenochrome and destroys its effect. Addition of ATP is necessary to start the reaction in this system. Inhibition sets in immediately but, in agreement with the former findings (1), higher concentrations of Adr. are needed, depending on the amounts of ATP present.

[2] Abbreviations used: ATP = adenosinetriphosphate; ADP = adenosinediphosphate; ATPase = enzyme splitting first labile P-group of ATP; apyrase = adenylpyrophosphatase, enzyme splitting both labile P groups; pyro-P = labile P of ATP and ADP; HDP = hexosediphosphate; HMP = hexosemonophosphate; Adr. = adrenochrome.

With such a centrifuged extract one can easily distinguish between the turnover of free sugar, HMP, and HDP; it then becomes immediately apparent that only the first two are inhibited by concentrations in the range of 10^{-5} to 10^{-4} M Adr., while the turnover of HDP is inhibited only 20% even with 4×10^{-4} M. For 90% inhibition about 3×10^{-3} M Adr. are needed for the latter (Table I and Fig. 1).

Although some difference often exists in the sense that glycolysis of HDP is less sensitive, because fewer transphosporylations are necessary

TABLE I

Adrenochrome Inhibition in Brain Extract

(0.3 cc. of 5 times diluted extract in 1.15 cc. total vol. at 38°C.)

No.	Time	10^{-5} M Adr.	10^{-5} M ATP	Sugar added	mm.³ CO_2		Inhibition
					Without Adr.	With Adr.	
	min.						*Per cent*
293	60	20	10	g[a]	338	10	97
	60	20	10	HMP	342	15	96
	60	20	10	HDP	162	131	20
	60	2	10	g	338	320	5
	60	2	10	HMP	342	328	5
294	60	4	3	g	308	150	52
	60	4	3	HDP	218	218	0
	60	2	3	g	308	239	30
296	50	10	10	g	365	291	20
	50	10	2	g	252	159	37
	50	10	0.8	g	187	44	76
298	60	20	10	HDP	198	187	6
	60	40	5	·HDP	192	154	20
	60	20	5	HDP	192	177	8
299[b]	40	40	10	HDP	365	280	23
	40	20	10	HDP	365	365	0
353	55	20	10	g	304	8	97
	55	2.5	10	g	304	91	70

[a] g = glucose.
[b] Complete homogenate.

for its turnover, such a marked difference leads immediately to the conclusion that the enzymes hexokinase and phosphohexokinase are the most sensitive toward Adr.
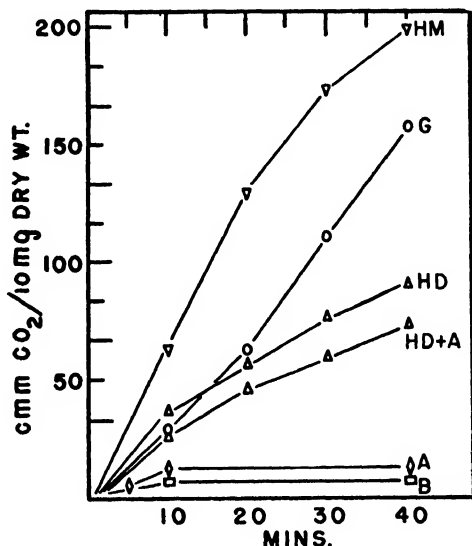
Fig. 1. Turnover of glucose (G), HMP, and HDP and inhibition by adreno-chrome. Centrifuged extract of brain homogenate. (No. 293.) A. ◇ Glucose and HMP turnover (same points) in the presence of $2 \times 10^{-4} M$ Adr. △HD + A = HDP turn-over in the presence of $2 \times 10^{-4} M$ Adr. The other curves without Adr. ○G = glu-cose; ▽HM = HMP; △HD = HDP; B = Blank.

That the reactions:

(1) glucose+ATP→hexosemonophosphate+ADP   (Hexokinase)

(2) hexosemonophosphate+ATP→HDP+ADP   (Phosphohexokinase)

are specifically inhibited can be proved by measuring the transphos-phorylation directly in the presence of NaF. This is shown in Table II and in Figs. 2 and 3. Inhibition of transphosphorylation is always ac-companied by activation of dephosphorylation. This is really caused by the check of rephosphorylation and not by a true activation of ATPase, as can be seen if sugar is omitted from the system; in this case adrenochrome has no effect on the speed of dephosphorylation (Table II, No. 386).

The black areas of Figs. 2 and 3 give the amounts of pyro-P present at any one time. The dotted parts of the blocks represent the amounts

TABLE II

Transphosphorylation and Dephosphorylation of ATP in the Presence of Adrenochrome
$(6 \times 10^{-2}\ M$ NaF. Total vol. 1.4 cc., 38°C.)

No.	Preparation	Sugar (g =glu-cose)	Adrenochrome γ	$10^{-5}\ M$	Time	Pyro-P ATP	Increase of inorganic P	Change of 7-min. P
					mins.	γ	γ	γ
306	0.5 ext.	—	----		0	67.0	—	—
	1:3.6	—	—		10	41.6	21.2	— 4.3
		g	—		10	30.4	6.2	−30.2
		g	40	16	10	28.3	12.0	−26.4
		g	120	48	10	26.6	20.0	−20.2
		HMP	—		10	44.6	4.3	−16.0
		HMP	120	48	10	42.6	15.9	— 4.0
311	0.5 ext.	--	—		0	79.0	—	--
	1:3.6	--	—		10	7.2	74.5	— 3.0
		g	---		10	25.0	40.8	−13.0
		g	120	48	10	10.8	61.0	— 7.0
312	0.5 ext.	—	—		0	72.5	—	—
	1:4	—	--		10	49.5	20.6	— 3.4
		g	—		10	32.6	7.4	−33.6
		g	40	16	10	40.0	3.4	−31.0
		g	120	48	10	32.9	19.2	−21.6
		HMP	—		10	56.5	7.0	−11.5
		HMP	40	16	10	51.2	11.3	−10.5
		HMP	120	48	10	53.1	16.4	— 7.2
345	0.5 ext.	—	—		0	70.0	—	—
	1:5	—	---		15	44.8	22.0	— 2.5
		g	—		5	42.1	5.9	−21.2
		g	—		10	34.7	6.6	−28.0
		g	120	48	5	43.8	9.0	−17.4
		g	120	48	10	40.5	12.4	−17.4
386	0.2 hom.	—	—		6	188	—	—
	1:5	--	—		2	125	64.4	— 1.4
		—	160	60	2	127	61.0	—
		—				135	48.0	— 5.0
	0.2 hom.	—	—		6	135	48.0	— 5.0
	1:5	—	160	60	6	136	50.5	— 1.5
	0.1n NaF							
	0.2 ext.	—	—		10	166	12.9	— 9.0
	1:5	---	160	60	10	172	12.9	— 3.0
	0.1n NaF							

transphosphorylated, measured by the decrease of 7-minute P, while the lower white parts of the blocks represent the amounts dephosphorylated by ATPase. The more transphosphorylation, the less dephosphoylation occurs and *vice versa*.
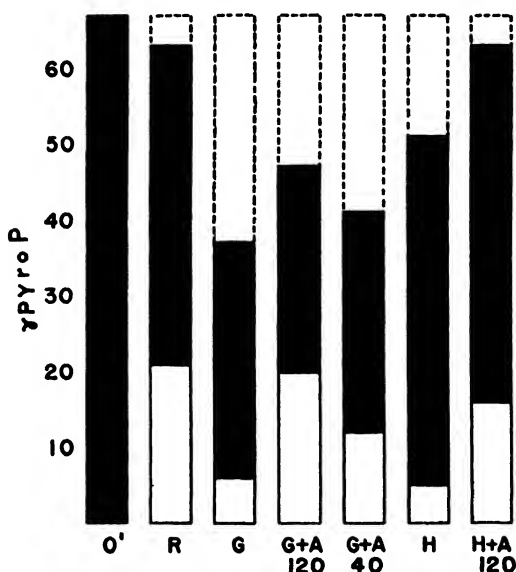
FIG. 2. Transphosphorylation between ATP and glucose or HMP in absence and presence of Adr. with 0.07 N NaF. Black areas of the blocks give the pyro-P present. Areas enclosed by dotted lines give the amounts of ATP which have disappeared by transphosphorylation to sugar. Areas enclosed by continuous lines at the bottom give the amounts of ATP which have disappeared by dephosphorylation. (No. 306) Incubation for 10 min. at 38°C., total vol. of 1.4 cc.; col. 0′ equals the ATP at 0 min. R = incubation with Ringer, G with glucose, H with HMP. G + A 120 = glucose with 120 γ Adr. G + A 40 = glucose with 40 γ Adr. H + A 120 = HMP with 120 γ Adr.

For higher accuracy two corrections should be applied which necessitate additional measurements and which for the sake of simplicity we have omitted since they would not change the trend of the experiments:

(1) There is some excess dephosphorylation caused by ordinary phosphatases; this can be determined by testing the system in the absence of ATP, using, *e.g.*, glucose-6-phosphate as substrate. This unspecific dephosphorylation is small compared to the action of the ATPase, roughly one-tenth of it, as was found earlier (4).

(2) Acid hydrolysis for 7 min. splits not only the labile P of ̄ATP but also about 33% of the HDP and a very small part of HMP (Robison ester), which is formed from glucose. For exact correction the values for 15 and 30 min. hydrolysis would have to be determined (cf.

FIG. 3. Transphosphorylation from ATP to glucose with and without Adr. Two time intervals (No. 345), 5 and 10 min. at 38°C. Adr. $4.5 \times 10^{-4} M$ (120 γ/1.4 cc.) 0′ = ATP at start. 10′ = Ringer after 10 minutes. 5′ and 10′ = glucose after 5 and 10
　　　　R　　　　　　　　　　　　　　　　　G　　G
minutes. 5′ and 10′ = glucose after 5 and 10 minutes with 120 γ Adr.
　　G + A　　G + A

Lohmann, 5) and the amounts of these esters computed from these determinations. We may assume that practicall} only HMP forms from glucose and that this does not need a correction.[3] On the other

[3] Earlier results with hexokinase from brain and other tissues had shown that in the absence of cozymase (*i.e.*, of the oxidation-reduction system) practically nothing but HMP is formed at first with an excess of glucose. This is probably because the concentration of HMP is too low compared with that of sugar to compete for the available ATP.

To make sure that the same thing holds under our conditions two additional experi-

hand, only HDP forms from HMP. Because the amount should be equivalent to the labile P of ATP which disappears, the dotted areas of the blocks II of Fig. 2 are only two-thirds of the true loss of pyro-P. One-third of the loss is veiled by the appearance of HDP. Although in this case the transphosphorylation is 50% greater than appears from the drawings we have refrained from correcting them in this way.

The effective concentrations of Adr. are at least ten times as high as those which give an appreciable (in some cases, complete) inhibition of glycolysis. There are probably three reasons for this: (1) Three to four times as much ATP is needed for the transphosphorylation experiments as for the glycolysis. This appreciably diminishes the effectiveness of Adr.

(2) In glycolysis of glucose the inhibition of hexokinase and phosphohexokinase are additive.

(3) Other glycolyzing enzymes are inhibited, too, although to a lesser degree, as can be seen from the slight inhibition of HDP.

The inhibition by Adr. is only marked when the hexokinase has its normal activity. In acetone powder extract, the transphosphorylation to glucose is not measurably inhibited and that to HMP, only slightly. As is shown in the preceding paper, in this preparation free sugar is only slowly glycolyzed and only $\frac{1}{4}$ of the HMP is quickly glycolyzed, owing to the lack of ATPase.

In extract of transplanted sarcoma the inhibition by Adr. is somewhat less. Here only HMP and HDP are strongly metabolized. This

ments were made, where the disappearance of glucose was compared with the diminution of the 7 min. P of ATP. Glucose was determined by the method of Nelson (11) where the phosphate esters are removed by barium hydroxide. In this case a low concentration of glucose (about 400 γ/cc.) and a higher concentration of ATP (about 100 γ pyro P/cc.) are required in order to obtain a sufficiently large change of the glucose content. This decreases somewhat the effectiveness of the Adr. concentrations used above.

The directly measured disappearance of sugar in 10 min. experiments made exactly like experiment 306 of Table II was 222 γ in the absence of Adr. and 164 γ with 5.10^{-4} Adr., while the 7 min. P decreased by 38.5 γ corresponding to 224 γ sugar without Adr., and by 29.8 corresponding to 172 γ sugar with Adr. In a second experiment the disappearance of glucose was 183 γ without and 128 γ with Adr. and the change of 7 min. P again corresponded to this. The inhibitions here were 30%.

This confirms the contentions: (1) that only HMP and no HDP forms, because otherwise more 7 min. P would disappear than the equivalent of sugar, and (2) that the inhibition concerns the hexokinase itself.

must be ascribed to a preferential damage to the hexokinase in preparing the homogenate. Glycolysis of HMP in sarcoma extract is inhibited 60% by 16×10^{-5} M Adr. as compared to 95% in brain extract with the same amount of ATP (Table III). Accordingly, only a slight in-

TABLE III

Adrenochrome Inhibition of Sarcoma Extract

No.	Time	$10^{-5} M$ Adr.	$10^{-5} M$ ATP	Sugar added	mm.^{3} CO_2		Inhibition
					Without Adr.	With Adr.	
	min.						*Per cent*
320	40	16	10	HMP	480	188	60
	40	16	10	HDP	278	226	20

hibition of phosphohexokinase could be observed by measuring the transphosphorylation.

In a paper by Wajzer (7), the author found with frog muscle pulp that Adr. inhibited lactic acid formation by blocking the phosphorylation of HMP to HDP. Because glycolysis in frog muscle starts with glycogen and not with free sugar, it is quite conceivable that in this case the phosphohexokinase is the only enzyme responsible for the inhibition of lactic acid formation. But in brain tissue it follows from our findings that hexokinase and phosphohexokinase are inhibited to nearly the same degree.

Some other quinones were tested by us in connection with the inhibition by Adr. p-Quinone in 5.5×10^{-5} M concentration inhibits glycolysis of glucose in brain extract about 80%, that of HDP 30%. The o-naphthoquinones were about 10 times as active and showed inhibitions similar to those recently described by Kuhn and Beinert (8) for carboxylase of yeast. 1,2-Naphthoquinone itself, and 1,2-naphthoquinone-4-sulfonate, as well as 1,2-naphthoquinone-4,6-disulfonate, showed identical inhibitions; 5×10^{-6} M inhibits turnover of glucose 80%, and of HMP 100%; 3×10^{-6} M inhibits HMP 90%. On the other hand, HDP is inhibited by 5×10^{-6} M only 30%, and by 1.5×10^{-5} M, 80%. This difference between HDP and free sugar and HMP is not so great as with Adr., and although this difference indicates an inhibition of hexokinase, this is surely not the only enzyme attacked. HDP does not need any hexokinase or phophohexokinase for its turnover.

The turnover of the extract of acetone powder is less inhibited, but 4×10^{-6} M inhibits HMP 30%, while transphosphorylation with excess ATP is inhibited 70% by 5×10^{-5} M 1,2-naphthoquinone.[3]

Finally, it may be mentioned that purified hexokinase from yeast (9, 10) is not inhibited by Adr.

2. Respiration and Glycolysis of *Trypanosoma equiperdum*[4]

Since adrenochrome inhibits glycolysis of tissue extracts, it seemed of interest to study its effect on living unicellular organisms. *Trypanosoma equiperdum* were chosen for this purpose. A suspension of trypanosomes was obtained from the blood of experimentally infected mice. The blood from 6 mice was collected in 20 cc. of Tyrode's solution containing heparin to prevent coagulation. Red cells were removed by centrifuging at low speed for 3 minutes. The number of organisms in the suspension was determined by hemocytometer counts.

Respiration and glycolysis were measured in the Warburg apparatus at 37.5°C. Two cc. of the suspension, containing about 50 million trypanosomes, were added to each flask. For respiration studies, air was used as the gas phase and carbon dioxide was absorbed with 10% KOH. For glycolysis measurements, 0.1 cc. of 0.154 M NaHCO$_3$ was added and the gas phase was 95% N:5% CO$_2$. After equilibration, adrenochrome was added from the side arm in 0.1 cc. of Tyrode.

From Table IV it is apparent that the respiration and glycolysis of trypanosomes are inhibited by adrenochrome at 14×10^{-5} M. This concentration also immobilizes aerobically, while anaerobically about 56×10^{-5} M concentration was required. Thus, adrenochrome inhibits the metabolism of trypanosomes at about the same concentration at which it inhibits glycolysis in brain extracts.

As an example of quinones which might have the inhibitory action of adrenochrome, the effect of 1,2-naphthoquinone-4-sulfonic (K salt) on the respiration, glycolysis and mobility of trypanosomes was also studied. The data in Table V show that this quinone inhibits respiration and immobilizes the trypanosomes at a concentration of 18×10^{-5} M. Slightly higher concentrations are required anaerobically.

[3] The irregular increase in blood sugar with 1, 2-naphthoquinone-4-sulfonate found by Brückmann and Wertheimer (12) is probably caused by this inhibition of hexokinase.

[4] We are indebted to Dr. R. J. Schnitzer of the Chemotherapy Department, Hoffmann-La Roche, Inc., who supplied the trypanosomes for these experiments and performed the *in vivo* tests.

TABLE IV

Effect of Adrenochrome on the Respiration and Glycolysis of Trypanosoma equiperdum

Adrenochrome concentration 10⁻⁵ M	Control mm.³ᵃ	Respiration adrenochrome			Control mm.³ᵃ	Glycolysis adrenochrome		
		mm.³ᵃ	Inhibition	Motility		mm.³ᵃ	Inhibition	Motility
			Per cent				*Per cent*	
5.6	38	39	0	Mᵇ	60	58	3	M
5.7	40	47	0	M	58	56	4	M
14	40	45	0	M	51	54	5	M
14	37	13	65	Imm.	52	36	31	M
28	34	10	70	Imm.	44	31	30	M
28	38	12	58	Imm.	52	31	40	M
56					44	14	68	Imm.

ᵃ mm.³ = cubic millimeters of oxygen or carbon dioxide/hr./50 million trypanosomes. ᵇ M = Motility intact.

TABLE V

Effect of 1,2-Naphthoquinone-4-Sulfonic Acid (K Salt) on the Respiration and Glycolysis of Trypanosoma equiperdum

1,2-Naphtho-quinone-4-sulfonic acid (K salt) concentration 10⁻⁵ M	Respiration				Glycolysis			
	Control mm.³ᵃ	Naphthoquinone			Control mm.³ᵃ	Naphthoquinone		
		mm.³ᵃ	Inhibition	Motilityᵇ		mm.³ᵃ	Inhibition	Motility
			Per cent				*Per cent*	
3.6	33	29	12	M	33	31	6	M
7.2	33	28	15	M	33	22	34	M
7.2	40	32	20	M	46	36	22	M
14	40	20	50	M	46	40	12	M
18	38	10	74	Imm.	60	36	40	M
36	38	8	78	Imm.	60	20	66	Imm.
72					45	9	80	Imm.

ᵃ mm.³ = cubic millimeters of oxygen or carbon dioxide/hr./50 million trypanosomes. ᵇ M: Intact motility.

1,2-Naphthoquinone, therefore, has the same order of activity as adrenochrome. Since the concentrations of 1,2-naphthoquinone and adrenochrome which inhibit respiration and glycolysis are similar, it is probable that the mechanism of inhibition is the same.

The activity of adrenochrome and 1,2-naphthoquinone-4-sulfonic acid on trypanosomes *in vivo* was also studied. Six mice per group were infected intraabdominally with approximately 600,000 trypanosomes. The control mice showed trypanosomes after 24 hours and a heavy fatal infection developed after 2 days. A sublethal dose of adrenochrome (250 mg./kg.), administered on the first and second days, did not exert a significant activity. A group of mice treated subcutaneously with 500 mg./kg. of 1,2-naphthoquinone on the first and second days remained free of blood parasites for 48 hours, but 4 of the 6 mice died of toxic effects. The 2 remaining mice first showed trypanosomes on the third day and died on the fifth day. Another group, treated with 250 mg./kg. daily for 4 days, remained free of trypanosomes for 24 hours; the first parasites were observed on the 2nd day and increased in number on the 3rd day. The mice died of trypanosomiasis.

Since adrenochrome did not have an antitrypanosomal activity *in vivo* and 1,2-naphthoquinone sulfonic acid had only an inhibitory effect which delayed the infection for 24–48 hours, while they exerted strong antitrypanosomal activity *in vitro*, it is probable that the quinones were inactivated in the animal body before they could affect the trypanosomes.

ACKNOWLEDGMENT

SUMMARY

In the first part of the paper it is shown that the inhibition of glycolysis of brain extract by adrenochrome is mainly due to inhibition of hexokinase and phosphohexokinase. *o*-Naphthoquinone is about five times as active and acts similarly; besides, it also inhibits the other enzymes of the glycolytic cycle.

In the second part adrenochrome and 1,2-naphthoquinone-4-sulfonic acid are tested on trypanosomes. Adrenochrome inhibits in about the same concentrations as in brain extract, but naphthoquinone in somewhat higher concentrations, the respiration, glycolysis and motility of trypanosomes *in vitro*. They do not exert an appreciable effect *in vivo*.

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An *in vitro* Bioassay for Intermedin [1]

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INTRODUCTION

In 1946 Wright (1) described an *in vitro* method for the detection of intermedin, the chromatophorotropic hormone of the *pars intermedia*. Chromatophore expansion in isolated frog skins immersed in physiological solutions containing pituitary extract was measured by observing the change in light transmission by means of a suitable colorimeter.

The convenience, objectivity, and sensitivity of the Wright technique suggested its possible wide application if a means of obtaining data of quantitative significance were available. Accordingly, a study has been made of the factors which influence the response of isolated pieces of frog skin to intermedin.

MATERIALS AND METHODS

Intermedin Solutions

Whole pituitary glands of sheep were macerated in a Waring blendor and twice extracted with hot 0.5% acetic acid. The extract was filtered and stored in the refrigerator. It is stable for weeks unless contaminated with mold or bacteria.

Skins from the thighs of 30–70 g. frogs (*Rana pipiens*) were removed, cleaned (without scraping) of adherent muscle and the larger blood vessels, and fitted over the aperture of the bakelite frame A shown in Fig. 1. Frame B is then fitted into place, and the two firmly clamped together by 4 machine screws, the brass pins in B holding the skin properly positioned. Excess tissue is then trimmed, and the frame placed in a glass or plexiglas cell which fits into the cell-holder of an industrial model Klett-Summerson photoelectric colorimeter. The zero adjustment is made without the cell, and with the shutter pulled out. The shutter is pushed in when the skin is in position, bringing the reading within the instrument's working range. For the experiments described here, a red filter (No. 66) was found most satisfactory.

In a typical experiment, the cell is first filled with amphibian Ringer's solution. As shown in Fig. 2, an immediate decrease in absorption occurs, corresponding to
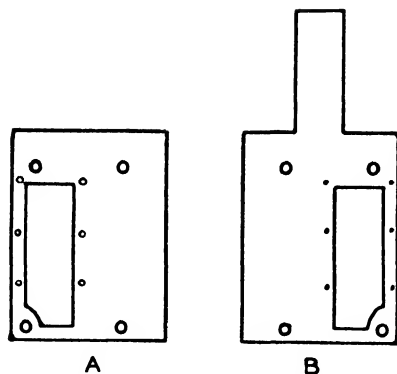
FIG. 1. Bakelite frames for holding frog skins.

spontaneous contraction of the chromatophores. The time necessary for the absorption to reach a minimum value (the base line) is variable, and may be shortened by using light-adapted animals. Occasionally, one or two preliminary washings are required to effect maximal contraction. If the Ringer's is now replaced by a (Ringer) solution of pituitary extract, a rapid increase of absorption results. This reaches a maximum in 1–2 hours, depending somewhat on the concentration. Replacing the pituitary preparation by fresh Ringer's causes the absorption to again decrease. The entire cycle can be repeated 2 or 3 times with the same skin.
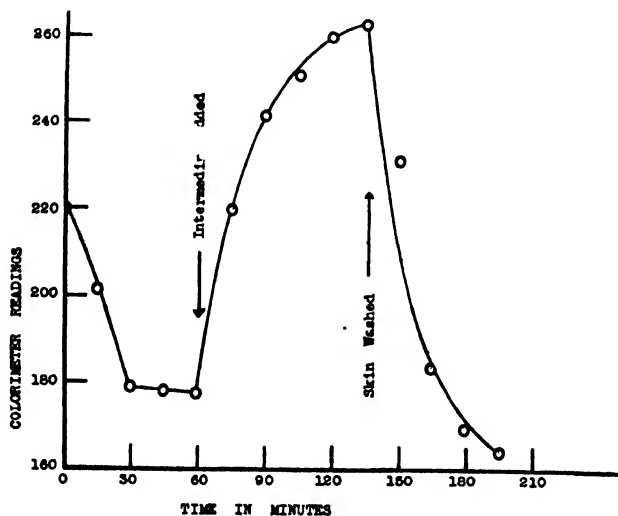
FIG. 2. Changes in light transmission of frog skin following: (a) spontaneous melanophore contraction; (b) melanophore expansion under influence of pituitary extracts; and (c) contraction following removal of extract.

In preliminary experiments, attention was directed toward ascertaining whether or not a relationship existed between extract concentration and absolute response or rate of response. It was immediately apparent that the response of different pieces of skin, from the same as well as from different animals, to the same concentration of extract, varied widely. The largest responses were observed at concentrations corresponding to 250 γ fresh tissue/ml.; solutions of higher, as well as lower, concentrations were generally less effective. Measurable responses, however, were observed using dilutions corresponding to 1–2
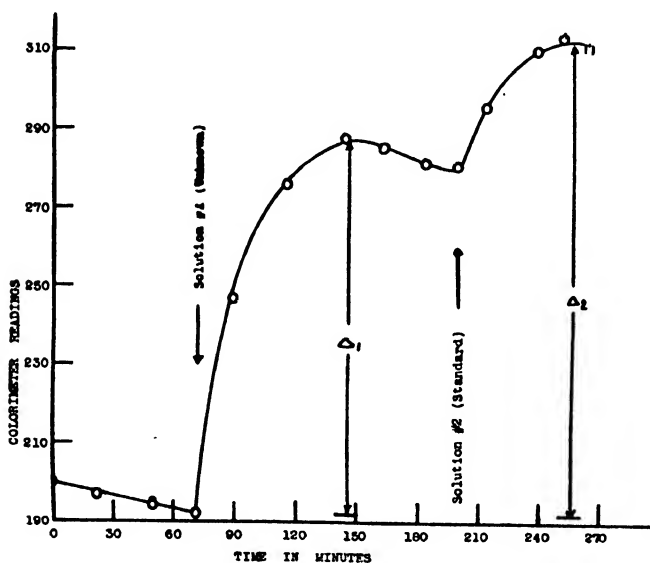
Fig. 3. Changes in light transmission of frog skin effected by unknown (Δ_1) and by standard pituitary extract (Δ_2).

γ/ml. fresh tissue. Nowhere within the range covered did there seem to be a consistent, reproducible relation between response and concentration, nor was a regular relation discernible between the rate of response and the concentration of pituitary substance.

In the next series of experiments, an attempt was made to eliminate the uncertainty due to the variable response of individual skins by measuring, as a function of concentration, the ratio of the response to the maximum of which each skin was capable. A series of skins were treated with various dilutions of pituitary extract (Fig. 3) and the

changes in transmission (Δ_1) recorded. All the skins were then subjected to the same concentration of extract; the difference between the second maximum and the original base line is recorded as Δ_2. Since it had been observed that maximal responses were obtained using solutions containing the equivalent of 250 γ/ml. of tissue, this was at first selected as the "standard" concentration. It was found that the ratio of the two responses (Δ_1/Δ_2) was proportional to log C (C = concentration of solution No. 1) when the latter was in the range 1–10 γ/ml. tissue equivalent, but considerable scattering of the data was apparent. It seemed likely that the uncertainty might partly be due to variation in skin sensitivity, which would be eliminated by using a "standard" closer to the working range. As a matter of fact when C = 10 γ/ml. tissue equivalent was selected as the standard, a good semilogarithmic relation between dosage and relative response was obtained.

For the final standardization, a solid intermedin preparation was made by acetone precipitation of the pituitary extract. The dried precipitate contained most of the intermedin activity, and was about 10 times as active as fresh tissue. Using solutions of this material at 1.0 γ/ml. as the standard, 50 determinations of Δ_1/Δ_2 were made, in which the concentration of solution No. 1 was between 2.0 and 0.06 γ/ml.

The results are summarized in Table I and Fig. 4. For the present an intermedin unit is tentatively defined as 1.0 γ of the preparation described above.

TABLE I

Concentration-Response Data for Frog Skins in vitro

Solution No. 1	Solution No. 2	No. of determinations	Av. Δ_1/Δ_2	Range of Δ_1/Δ_2
units/ml.	*units/ml.*			
2.0	1.0	4	1.18±.10	.98–1.35
1.0	1.0	7	0.95±.04	.92–1.02
0.50	1.0	14	0.82±.06	.74– .92
0.25	1.0	9	0.57±.06	.49– .63
0.125	1.0	11	0.42±.05	.35– .50
0.0625	1.0	5	0.21±.02	.17– .26

As an example of the application of the method, assay data for 6 determinations of the intermedin content of USP posterior lobe reference standard B3801 are given in Table II. The powder was extracted in the usual manner with hot dilute acetic acid.

The observations that the *in vitro* effect of intermedin, like the *in vivo* effect, is reversed by adrenaline, suggested a possible means of setting up an absolute standard for the melanophore hormone. Using the skin as an indicator, so to speak, it seemed possible that an intermedin unit might be defined in terms of the quantity which is parti-
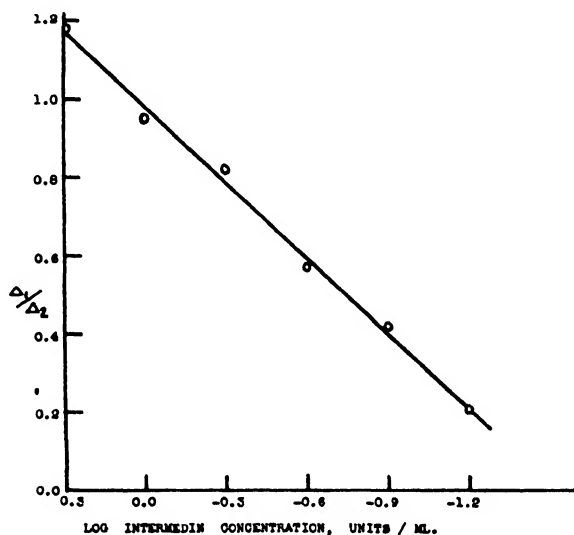
FIG. 4. Relation between relative response (Δ_1/Δ_2) and intermedin concentration (see Table I).

TABLE II

Intermedin Assay of USP Posterior Lobe Powder (Reference Standard B3801)

Sample No.	1	2	3	4	5	6
Equiv. powder conc., γ/ml.	0.025	0.0125	0.00625	0.025	0.0125	0.00625
Δ_1	49	28	26	82	37	10
Δ_2	107	96	121	141	111	75
Ratio, Δ_1/Δ_2	0.46	0.29	0.22	0.58	0.33	0.13
Log C	−0.80	−1.06	−1.17	−0.62	−1.00	−1.31
C (Units/ml.)	0.16	0.087	0.068	0.24	0.10	0.049
Intermedin content of powder, Units/γ	6.4	7.0	10.9	9.6	8.0	7.8

Average, units/γ: 8.3 ± 1.5.

ally (*e.g.*, 50%) or completely inhibited by a definite quantity of crystalline adrenaline hydrochloride. Experiments in this direction have thus far proved disappointing; the critical adrenaline: intermedin ratio seemed to vary over a comparatively wide range. It would appear that the relative sensitivity of skins to the two hormones varies considerably.

Specificity

In preliminary experiments, designed to assess the specificity of the *in vitro* assay, the effects of acetic acid extracts of various rat tissues were studied. A positive response may readily be obtained with extracts of rat pituitary containing 0.1 γ/ml. tissue equivalent. Similar responses are obtained only with 100–1000 times more concentrated extracts of rat adrenal, thyroid, liver, spleen and kidney, while rat serum affords only a minimal response at 1:5 dilution. It is also pertinent to note that the well-known potentiating effect of dilute alkali upon intermedin (2) can readily be demonstrated by the method described here.

Sensitivity

It can be observed (Fig. 4) that positive, measurable responses are elicited by concentrations as law as 0.06 units/ml. It is difficult to compare this figure with the sensitivity of other assay techniques, because no preparation of standard potency is available. A rough comparison can, however, be made, if it is assumed that the chromatophore hormone content of standard posterior pituitary powder is approximately constant from one preparation to another.[2] Table II shows that the USP powder used by us averages 8.3 units/γ. Half-maximal response (Fig. 4, $\Delta_1/\Delta_2 = 0.5$) corresponds to a concentration of 0.18 unit/ml.; this response would thus be given by 0.022 γ/ml. of USP powder. In Table III this sensitivity is compared with that of three other methods for which comparable data are available.

The procedure described above has been found admirably suited to studies dealing with the purification of the chromatophore hormone. It has been in routine use in our laboratory for only a few months, so we are as yet unable to comment about the effect of seasonal variation.

[2] Landgrebe and Waring (3) have shown that the melanophore hormone content of the Second International Standard P.L. powder is within 10% that of the First International Standard.

TABLE III

Comparative Sensitivity of Intermedin Assays

Test object	Criterion of response	Micrograms P.L. powder for 50% response	Reference
Denervated fin of *Fundulus*	Darkening of denervated area	30[a]	Abramowitz (4)
Hypophysectomized *Rana*	Duration of response of leg melanophores	15[b]	Calloway *et al.* (5)
Hypophysectomized *Xenopus*	Melanophore index in web	0.625[c]	Landgrebe and Waring (3)
Isolated skin of *Rana*	Relative transmission decrease	0.022(γ/ml.)	This paper

[a] 50% response at 12–18°C.; there is a considerable temperature coefficient. Powder extracted with pyridine.

[b] Average time for complete contraction = 150 min. Powder extracted at pH 3.

[c] Average melanophore index of 2.5. First International P.L. standard, extracted with dilute HOAc.

However, the use of relative response as a criterion should at least minimize this factor.

SUMMARY

A method is described for the *in vitro* assay of intermedin, using pieces of excised skin of *Rana pipiens*. The criterion of activity is the ratio of the change in transmission of light through such a skin in response to an unknown as compared with that produced by a standard intermedin preparation. This ratio is linear with the log. of intermedin concentration. The method is sensitive to 0.01 γ/ml. of standard posterior lobe powder. Evidence relating to the specificity of the assay is given.

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Actinomycin A Produced by a Soil *Actinomyces* Different from *Actinomyces antibioticus* [1]

From a sample of unmanured soil an *actinomyces* (A-18), quite different from *Actinomyces antibioticus*, has been isolated which produces actinomycin A. This short note is intended to aid those who might encounter this organism in their search for new antibiotics.

Actinomyces A-18 has been tentatively identified as *Actinomyces parvus* Krainsky. It is distinct from *A. antibioticus* (1) known to produce actinomycin A, and from a culture (Strain 78) thought by Waksman *et al.* (2) to form an actinomycin-like substance. In contrast to these strains, A-18 does not blacken peptone or gelatin media. It is characterized by vigorous growth and sporulation on many media. The vegetative growth is bright yellow. The spores are white, becoming yellow. A profuse soluble yellow to yellow-orange pigment is produced. This is in striking contrast to the brown pigment produced by *A. antibioticus* and is a great aid in following the formation of the highly colored actinomycin A during growth of the cultures.

The optimum temperature for growth is about 25°C.; however, growth will take place at 15°C. and at 37°C. Conidia are spherical to laterally compressed, and are borne in chains. No spirals were observed. Abundant yellow vegetative and aerial mycelium and yellow soluble pigment are produced on nutrient agar. Abundant yellow growth and soluble pigment are produced in nutrient broth. Profuse yellow vegetative and aerial mycelium and orange pigment are produced on nutrient agar containing 5% glycerine. Moderate growth with white spores and soluble yellow pigment appear on glucose-asparagine agar. Poor spreading growth with white spores takes place on Czapek agar.

Moderately yellow vegetative mycelium, white spores becoming yellow, and soluble yellow pigment are produced on calcium citrate agar. Milk becomes alkaline and is digested. Gelatin is liquefied and a soluble yellow pigment is produced. Abundant vegetative growth takes place on potato; scanty white spores and little pigment are formed. Starch is hydrolyzed. There is no action on cellulose.

Actinomycin A is produced abundantly by this organism on a medium consisting of 5 g. Bacto-tryptone, 10 g. cerulose, 2 g. K_2HPO_4, 2 g. NaCl in 1,000 cc. distilled water. When grown at 26–27°C. in stationary layers with the addition of 2.5 g. agar/liter, the maximum production of actinomycin A is reached on the 6th to 8th day. At this time the organism has formed a yellowish-white surface-lace type of growth and the medium is bright orange in color. The pH of the medium at the time of harvest is 8.0–8.2. Using the streak test and *Staphylococcus aureus* as the test organism, the antibiotic activity of the culture assayed at harvesting time is about 2,000 dilution units/ml. of culture fluid. One dilution unit is the smallest amount of culture fluid/ml. of nutrient agar which will prevent growth of *S. aureus* for 24 hours at 37°C. The antibiotic activity of the culture fluid is 5–10 times greater against *Bacillus mycoides*, *B. subtilis* and *Sarcina lutea* than it is against *S. aureus*.

The purification of actinomycin A was carried out as previously described.[3] The crude culture, harvested at the peak of production, was extracted in a continuous extractor with ether, and the ether extract evaporated to dryness. The bright orange-red residue was extracted exhaustively with petroleum ether to remove oily substances. The residue was then dissolved in benzene and poured through a column of Brockmann alumina, washed with a small amount of benzene, developed with acetone-benzene (15:85), and finally washed into the percolate with acetone-benzene (30:70). The colored fractions of the percolate were evaporated to dryness and dissolved in a small amount of acetone-ether (1:10) and put in the refrigerator. After standing a few hours, garnet colored pyramids appeared which melted, after a second recrystallization from the solvent, at 249–251°C. with decomposition. Since the melting point and other characteristics coincided with the properties reported for actinomycin A, no further analyses were made.

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The Effect of Growth Hormone on the Incorporation of S^{35} of Methionine into Skeletal Muscle Protein of Normal and Hypophysectomized Animals [1]

It has been shown that the administration of purified hypophyseal growth hormone to hypophysectomized and normal animals initiates an increase in body weight and a correspondingly greater nitrogen retention (1). However, it is not clear whether this observed nitrogen retention is due to increased protein anabolism and/or to decreased protein catabolism. The experiments reported below favor the explanation that growth hormone exerts its effect by stimulating the incorporation of amino acids into proteins, that is, by actually accelerating protein anabolism. The uptake of S^{35} (administered as methionine) by the skelectal muscle proteins of normal mice and hypophysectomized rats was studied, as influenced by the administration of purified growth hormone.

Male rats, weighing about 200 g., were hypophysectomized. Five days later, 1.5 mg. of growth hormone was administered intraperitoneally for 2 successive days. On the third day, the dose of hormone was increased to 2 mg. (given 8 hours before sacrifice). During these 3 days, the untreated animals, having received no hormone, lost weight, while the growth hormone-treated animals gained several grams. All animals were fasted 6 hours, injected *via* the jugular vein with a parenamine-radiomethionine mixture,[2] and sacrificed 6 hours later. The hormone was prepared as described by Li and Evans (2).

Normal male mice, weighing about 20 g., were injected intraperitoneally with 3 mg. of growth hormone over a 3 day period. The last injection was made 4 hours before the amino acid mixture was administered by tail vein. Food was removed from the animals 6 hours before the intravenous injection of the parenamine-radiomethionine mixture, and the mice were sacrificed 4 hours later.

TABLE I

Incorporation of S^{35} of Methionine into Skeletal Muscle Protein [a]

Treatment	Weight gain[b] g.	Specific activity $\times$ 100 $\times$ wt. of animal in kg. / Administered dose S^{35}
Normal mice	0.3±0.1	0.034±0.001 (6)
Growth hormone-treated mice	1.0±0.1	0.046±0.002 (6)
Hypophysectomized rats	−4.0±0.9	0.20 ±0.02 (2)
Growth hormone-treated hypophysectomized rats	14 ±3.1	0.34 ±0.04 (3)

[a] Incorporation of methionine expressed in terms of a function of the specific activity $\frac{S^{35}}{S^{32}}$. Data are arithmetic means ± standard error of mean. Value in brackets indicates number of animals used.

[b] Weight gains are for the period of hormone administration (see text). Control and hormone-treated animals were of the same age and were selected to be of approximately the same weight at the time of administration of labeled methionine. Average weights were:

Normal mice	19.4± 0.2 g.
Growth hormone-treated mice	19.6± 0.2 g.
Hypophysectomized rats	190 ±11.3 g.
Growth hormone-treated hypophysectomized rats	198 ± 8.2 g.

[2] This mixture consists of a solution of parenamine (a protein hydrolyzate) with a content of 16 mg. total α-amino nitrogen and 36 mg. of methionine/ml. A small amount of labeled methionine (about 0.1 mg., possessing 16,000 counts/minute) was added to each ml. 0.16 g. of amino nitrogen was administered/kg. of body weight.

The method of analysis for S^{35} developed by Tarver (3) was employed. The protein from homogenized tissue was precipitated with 10% trichloroacetic acid and washed 4 times with 5% trichloroacetic acid. The separated protein was digested with Pirie's reagent and analyzed for sulfur by benzidine precipitation and subsequent titration with 0.05 N NaOH. The radioactivity of the sulfur was determined by precipitating it as barium sulfate and counting with a Geiger-Muller counter.

In Table I it is shown that the incorporation of sulfur from methionine into the protein of skeletal muscle 6 hours after its injection was increased about 70% by administration of growth hormone. In this initial time interval, when the incorporation of S^{35} into tissue protein increases sharply, protein catabolism can have little influence on the S^{35} concentration of the protein. Consequently, the specific activity of the labeled sulfur mainly is a measure of protein synthesis. Growth hormone does not promote protein synthesis in all body tissues; liver, for example, shows a reduction in amino acid uptake under its influence (4).

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Blood Group Substance from Intestinal Mucosa and its Precipitation with Borate

A polysaccharide fraction from phosphoesterase preparations from calf intestinal mucosa has been obtained (1, 2) by precipitation with borate. General procedure: A solution of about 1% concentration is 57% saturated with ammonium sulfate. Any precipitate is removed and 7 volumes of the solution are mixed with 1 volume of 0.2 M sodium

tetraborate. For quantitative precipitation the mixture is kept at low temperature for several hours and stirred to break up the gel which forms. The precipitate is dissolved in water, dialyzed, and precipitated with acetone and sodium acetate. The polysaccharide is purified by repetition of this procedure, and finally deproteinized by stirring with chloroform-amyl alcohol (3). The polysaccharide from calf intestinal mucosa was obtained homogeneous as shown by electrophoresis in phosphate buffer (2); it contained (corrected for 7.3% volatile matter) 5.33% N, 51.0% reducing sugar on hydrolysis (1) and had $[\alpha]_D^{20}$ of $+9°$. A relation between this polysaccharide and the specific blood group substances is suggested. Furthermore, the small intestine of man was found to be (4) rich in these substances and Blood Group A substance from hog gastric mucin was found to be (5) more viscous in the presence of borate, a property also shown by the polysaccharide from calf intestinal mucosa (2).

By using the borate precipitation, a fraction behaving like that from intestinal mucosa was obtained in 10% yield from a good source of Blood Group A substance, commercial hog gastric mucin (Wilson Labs. Type 1701-W). Without further treatment it was free of protein (chloroform-amyl alcohol test) and contained (corrected for 10.0% volatile matter) 4.65% N and 58.6% reducing sugar on hydrolysis.

Since the relation of the Type 14 *pneumococcus* polysaccharide to blood group substances is well established (6), Type 14 serum (horse) was used to demonstrate the relation of the polysaccharide from intestinal mucosa to the blood group substances. A sample of Type 14 serum[1] gave precipitates (7°) with Group A substance[2] and with the substance isolated from hog mucin by the borate procedure diluted 1:50,000, and with that from calf intestinal mucosa diluted 1:10,000. Cruder preparations from hog and dog intestinal mucosa also precipitated with this serum. The polysaccharide from calf intestinal mucosa in high dilution neutralized Blood Group A agglutinins.

These studies show that intestinal mucosa provides an additional source for the blood group substances and, most important, that the borate procedure provides a convenient and apparently specific method for their separation from proteins. Other polysaccharides,

[1] Supplied by New York City Department of Health, Bureau of Laboratories.

[2] A mixture of A and B substances supplied by Sharp and Dohme, L. Earle Arnow, Director of Medical Research.

however, may precipitate with borate also. These findings should be of additional interest in view of recent reports that the blood group and other polysaccharides interfere with certain viruses (7, 8).

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An Unusually Heat-Resistant Pectolytic Factor from Tomatoes [1]

Some time ago one of us (1) expressed doubt concerning the occurrence in tomatoes of a pectin-polygalacturonase (PG) (2) identical with the PG produced by molds. Recently MacDonnell, Jansen and Lineweaver (3), as well as Hills and Mottern (4), reported the presence of such an enzyme in tomatoes. Upon reinvestigating this subject we have found that a pectolytic factor indeed occurs in tomatoes (5) but that its action mechanism on pectic acid seems to be different from that of mold PG. In addition, a portion of this enzyme shows a rather uncommon heat resistance.

This pectolytic factor is present in the highly active tomato pectin-methylesterase preparations previously reported from this laboratory (6). The precipitates formed after the dialysis might be taken up in a saturated solution of NaCl at pH 5.5 or in a solution of 0.5 M Na acetate at pH 7.0. As an example of the activity of such a preparation,

1 ml. when mixed with 50 ml. of 0.2% pectic acid solution containing 0.1 M NaCl caused, at pH 4.5 and 30°C., the complete disappearance of pectic acid precipitable as calcium pectate in less than 15 minutes. The major difference between these reactions and the hydrolysis of pectic acid by mold PG is that, eventually, with the tomato enzyme a maximum reducing power in the range of 40–45% of the theoretical value is reached but additions of more tomato enzyme will not further advance the hydrolysis. Addition of mold PG will rapidly cause complete hydrolysis and nearly the theoretical 100% calculated reducing power. This seems to indicate that either different polygalacturonases are involved in the hydrolysis of polygalacturonic acids of different degrees of polymerization or that the linkages connecting the anhydrogalacturonic acid residues in pectic acid are not all equally available for the fissure (7).

Upon holding the tomato enzyme preparations in a boiling water bath there is an initial rapid loss of about 80% of the total pectolytic activity in the first 5 minutes, after which the loss in activity proceeds slowly, reaching 90% in 30 minutes and 95% in one hour. Activity measurements were based both on the time required to produce a 50% loss in calcium pectate and that required to produce a 10% increase in reducing power of the reaction mixture, and the results obtained by the 2 methods agree closely. On the basis of a preliminary comparison of the loss in calcium pectate per increase in reducing power, it appears that the modes of action of both the boiled and unboiled fractions on pectic acid are similar.

When the boiling times were 15–30 minutes, and using reaction mixtures similar to those noted above, the time required for the first measurable change in pectic acid varies from 2 to 4 hours with complete disappearance of calcium pectate-forming substances in from 20 to 50 hours. The observed activities are not due to infection by microorganisms, because thymol or toluol were used in all mixtures. The heat stability of the factor also permits the use of sterilized solutions of the enzyme in sterile reaction mixtures. The destruction of the pectic acid proceeds in these in the same manner.

When the original preparations are heated, a large amount of coagulated protein material precipitates out. The heat resistant factor is found to be present in the supernatant and may be separated from the coagulated proteins by centrifuging. The supernatant, containing the heat-resistant factor, gives a negative biuret and a negative Hopkins-Cole reaction. Both the heat-labile and the comparatively heat-resistant factor shows a sharp pH optimum at pH 4.5 and both can be inhibited by the addition of Ca^{++}, Cu^{++}, Nacconol (8), and Na diethyldithiocarbamate. The effects of Ca^{++} and Cu^{++} are likely to be in blocking the substrate rather than affecting the catalyst.

It is difficult to say at the present time whether or not the heat-stable factor is of "enzymatic character." An enzyme (9) and a plasma

coagulase (10) have previously been reported which show heat resistance of similar degree; however, they are both inactivated by trypsin and pepsin. Preliminary information on this heat-resistant factor seems to indicate that it is not easily inactivated by proteolytic enzymes.

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Intermediate in the Hydrolysis of Glycyldehydrophenylalanine by Dehydropeptidase I

The enzymatic breakdown of a dehydropeptide was considered by Bergmann and Schleich (1) to involve initial hydrolysis of the peptide bond with formation of an α,β-unsaturated amino acid, possibly in tautomeric equilibrium with the imino acid (2), this intermediate spontaneously decomposing to ammonia and the corresponding keto acid. Such imino acids had previously been assumed by Knoop (3) to be involved in the physiological equilibrium between α-amino and keto acids. However, the presence of such intermediates has never been demonstrated, presumably due to their great instability.

Studies reported here of the action of dehydropeptidase I on glycyldehydrophenylalanine carried out by following the disappearance of the characteristic ultraviolet absorption as described by Carter and Greenstein (4) provide evidence of the formation of such an inter-

mediate. At low substrate concentration and high enzymatic activity, the two-step reaction shown in Fig. 1 was observed. The enzyme, representing a 30-fold purification, was prepared from beef kidney by differential centrifugation following the observation that it was associated with particulates sedimentable at high speeds.

The rate of Step 1 (A-C), involving a shift in the peak from 275 to 295 mμ, is proportional to enzyme concentration, and close to that for hydrolysis of glycyldehydroalanine. Step 1 is stopped by heating to 90°C. and by 0.05 M KCN, an inhibitor of dehydropeptidase. No free am-
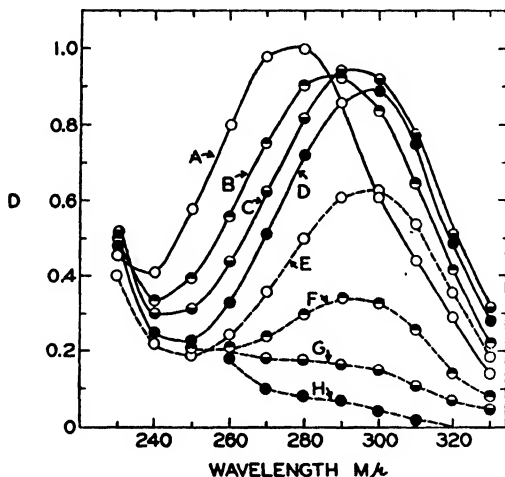
FIG. 1. Changes in absorption of glycyldehydrophenylalanine (5.7 $\times$ 10^{-5} M) incubated with dehydropeptidase I (0.00125 mg. enzyme N/ml. reaction mixture) at 38°C. in 0.02 M borate buffer pH 8.1. Times in minutes; A = 0, B = 10, C = 20, D = 40, E = 100, F = 205, G = 720, H = 1140. Ordinate D, measured optical density, 1 cm. cell.

monia was detected on direct Nesslerization at 20 minutes (C). The rate of Step 2 (C-H) is independent of enzyme concentration, unaffected by cyanide, greatly accelerated by heating to 90°C. or by acidification to pH 2, and slowed by alkali (pH 12) or by cooling. The absorption falls to a level approximating that of the reaction products, and increasing quantities of ammonia, up to the calculated yield, are found. Similar results were obtained with kidney homogenates but there was no spectral change with heat-inactivated enzyme or various inert proteins.

These facts are compatible with the concept that Step 1 involves the enzymatic hydrolysis of the peptide bond and that the resulting α-iminophenylpropionic acid (curve C) decomposes spontaneously in Step 2 to ammonia and phenylpyruvic acid. Attempts to isolate the intermediate are in progress. In similar studies with such aliphatic dehydropeptides as glycyldehydroalanine, the drop in absorption at all wavelengths paralleled formation of ammonia and followed a first order curve to complete hydrolysis, an indication that in these cases the second step is very rapid compared with the first. The phenyl residue thus appears to exert a marked stabilizing influence on the imino acid.

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Another Case of Reversible Oxygenation

Recently, two classes of coordinative cobaltous complex compounds (one as quoted in 1, 2, and 3; the other in 4) have been described, which share with hemoglobin the unusual property of reversibly combining with molecular oxygen without raising the oxidation level of the cobaltous to the cobaltic, or of the ferrous to the ferric state respectively. I wish to add another cobalt compound having the same property, which, to be sure, is not a new compound, but has been known for more than half a century, although it has never been appreciated in this respect. When a solution of cobaltous nitrate is mixed with ammonia and air is bubbled through, dark-brown crystals develop (5) which were formerly designated as oxycobaltiac. According to Werner and Mylius (5, 6) the following reaction takes place:

$$2[Co^{II}(NH_3)_6](NO_3)_2 + O_2 \rightleftharpoons$$
$$[Co(NH_3)_5 \cdot O_2 \cdot (NH_3)_5Co](NO_3)_4 + 2NH_3.$$
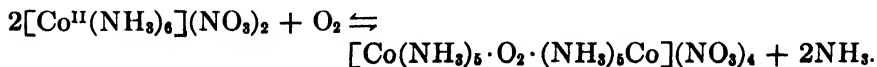
Since the coordination of ammonia with the cobaltous ion is rather weak, even in an excess of ammonia, one may just as well write:

$$2[Co^{II}(NH_3)_5H_2O](NO_3)_2 + O_2 \rightleftharpoons$$
$$[Co(NH_3)_5 \cdot O_2 \cdot (NH_3)_5Co](NO_3)_4 + 2H_2O.$$
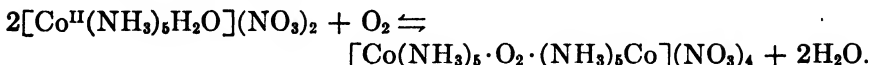
A binuclear cobaltous complex is formed, with O_2 as a bridge. Werner suggested that this reaction was reversible although he had no convincing experimental test. However, it can easily be shown that the coordination of oxygen is reversible, depending on the oxygen pressure, by the following experiment. Five grams of ammonium chloride and 10 cc. of strong aqueous ammonia are dissolved in water to a volume of 50 cc. This solution is freed from air by bubbling nitrogen through it in a gas washing bottle. To avoid too much loss of ammonia, the gas may be first passed through another solution of the same composition. Then 0.5 cc. of a 10% solution of cobalt chloride ($CoCl_2 \cdot 6H_2O$) are added. A very light pink color results. The solution is then shaken with air. Gradually, a brown color develops. On again bubbling nitrogen through the solution, this color fades out and eventually gives way to the original faint pink. This cycle can be repeated any number of times, even after several days. The rate of the reaction depends largely on the temperature. Each cycle may take half an hour at room temperature, much less at 40°C.

To be sure, when a concentrated solution of a cobaltous salt in excess ammonia has air bubbled through it for a very long time at a higher temperature, true cobaltic compounds are eventually produced, chiefly a mixture of roseo cobalt salts with some luteo cobalt salts; in the presence of charcoal (7) as a catalyst, the pure luteo cobalt salt is established. However, this is a very gradual, secondary process. Indeed, the "oxygenated" cobaltous ammine is even more stable than the oxygenated cobalt histidine complex.

It should, however, be borne in mind that this property is not quite general for all cobalt complexes. No reversible oxygenation was found in the case of ethylenediamine complexes, which are oxidized to the cobaltic state by exposure to air and cannot be restored to the cobaltous state by removal of oxygen. No reversible oxygenation can be expected, or is observed, for the hexacyanocobaltous ion which is directly oxidized to the cobaltic state even by water.

All cobaltous ammines are paramagnetic with 3 unpaired electrons. Feytis (8) found in 1911, and the writer can corroborate the fact,

that the solid "oxycobaltiac" nitrate is diamagnetic. The type of change in magnetic properties before and after oxygenation is the same as in the histidine compound (9) and different from the two types described by Calvin and associates (2, 3).

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The Specific Peptidase and Esterase Activities of Chymotrypsin [1]

In a recent report on the specific esterase activity of trypsin (1), qualitative observations of a like enzymatic activity of chymotrypsin were described. These suggested that the amino acid esterase activity may be a general attribute of proteolytic enzymes. The results of the present communication substantiate this conclusion for chymotrypsin, and analogous data on carboxypeptidase will be reported elsewhere (2).

Quantitative enzymatic measurements were made on glycyl-L-tyrosineamide and benzoyl-L-tyrosineamide for peptidase activity, and on benzoyl-L-tyrosine ethyl ester for esterase activity. Because of the limited solubility of the latter two substrates in aqueous buffers, chymotryptic hydrolysis of glycyltyrosineamide was

measured in methanol-water mixtures of varying composition, and that of the benzoyl derivatives in buffer solutions containing 30% methanol (pH 7.8, 25°C.). Reaction rates were determined as previously described (1).

The proteolytic coefficient (first order reaction constant/mg. enzyme N/cc.) for the chymotryptic hydrolysis of glycyltyrosineamide (0.025 M) was about 0.014 and decreased logarithmically with increasing methanol concentration to 0.0023 in 30% methanol. Benzoyltyrosineamide, previously reported to be resistant to chymotryptic hydrolysis (3), was found to be hydrolyzed more readily than glycyltyrosineamide. In 30% methanol and 0.025 M substrate solution, the calculated proteolytic coefficient was 0.45, $i.e.$, about 20 times higher than that of glycyltyrosineamide. The chymotryptic hydrolysis of benzolytyrosine ethyl ester in 30% methanol likewise followed apparent first order kinetics. In 0.025 M substrate solution, the proteolytic coefficient was more than 300 times higher than that of the corresponding amide, $i.e.$, about 16.

With both the ester and the amide of benzoyltyrosine a large increase of the calculated first order reaction constant with decreasing substrate concentration was noted, analogous to that previously observed for the splitting of carbobenzoxyglycylphenylalanine by carboxypeptidase (4). This can be fully accounted for by a shift of the equilibrium between free and combined enzyme during hydrolysis. If the latter is corrected for by integration of the Michaelis-Menten equation,[2] a reaction constant is calculated which is independent of enzyme and substrate concentration and characteristic only of the reaction system. For the hydrolysis of benzoyltyrosineamide in 30% methanol, this constant, k, is about 0.0022, and for benzoyltyrosine ethyl ester 0.72, the corresponding enzyme-substrate dissociation constants, K_m, being about 6.4×10^{-3} and 2.8×10^{-3}, respectively. Representative data are given in the table.

Acetyl-L-tyrosine ethyl ester is likewise hydrolyzed by chymotrypsin at a rapid rate, the quantitative results to be reported with the full description of the present experiments. Attempts to find a specific inhibitory peptide derivative for chymotrypsin are in progress. DL-1-Phenyl-1-acetaminobutanone-3 has been found to be inactive in this respect.

[2] We are indebted to Dr. Irving Klotz for a discussion of this problem. A more extensive experimental proof for the validity of this equation will be given elsewhere (4) (Elkins, E., and Neurath, H., manuscript in preparation).

TABLE I

Substrate	Conc.	Methanol	K^{1st}/E^a	k^b
	$10^{-2} M$	Per cent	10^{-2}	10^{-1}
Glycyl-L-tyrosineamide	5.00	0	1.0	—
	2.50	0	1.4	—
	2.50	12.5	0.7	—
	2.50	25.0	0.3	— -
Benzoyl-L-tyrosineamide	2.50	30.0	4.5	0.022
	1.73	30.0	6.8	0.023
Benzoyl-L-tyrosine	2.50	30.0	1560	7.2
ethyl ester	1.25	30.0	3800	7.2
	0.63	30.0	6610	7.2

^{a} K^{1st}/E is the first order reaction constant/mg. of enzyme N/cc.

^{b} k is calculated from the equation $v = -\dfrac{da}{dt} = \dfrac{k\,E\,a}{K_m - a}$, which, on integration[2], gives $2.3\,K_m \log a_o/a + (a_o - a) = k\,E\,t$, where K_m is the enzyme-substrate dissociation constant, a_o the initial substrate concentration and a the substrate concentration at time t, and E the total enzyme concentration in mg. N/cc.

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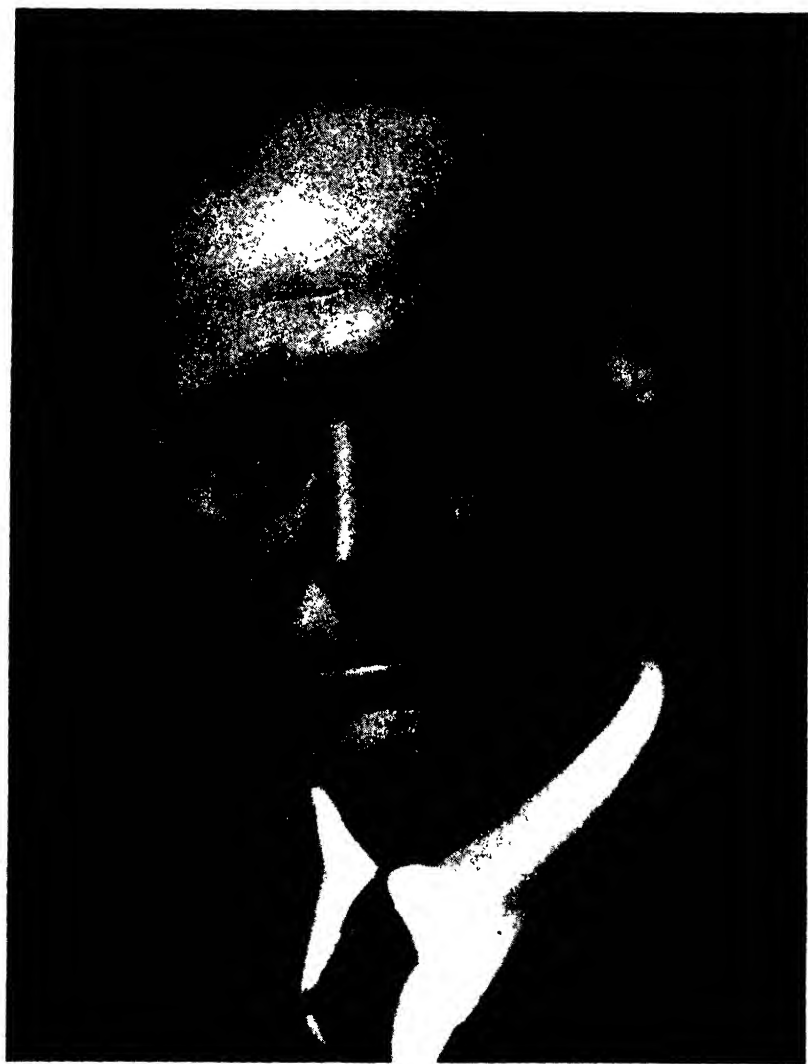
OBITUARY

Fred Conrad Koch

1876–1948

Fred Conrad Koch, Frank P. Hixon Distinguished Service Professor Emeritus of Biochemistry, The University of Chicago, and Director of Biochemical Research, Armour and Company, died suddenly on January 26, 1948, in Chicago. He will be missed by numerous friends and colleagues who, from intimate acquaintance with him, as well as with his work, came to regard him with admiration and affection.

He was born in Chicago May 16, 1876, and lived almost his entire life in this city, becoming one of its best known scientists. His early education was obtained in the public schools where he showed enthusiasm and aptitude for natural science. With the help of a high school scholarship and the encouragement of his grandfather, his father having died several years previously, he attended the University of Illinois, receiving the B.S. degree in 1899, specializing in chemistry. After graduation, he remained at Illinois as an instructor for two years. During the following seven years, he was employed as a Research Chemist by Armour and Company in Chicago. Always eager to learn and ambitious, he entered the University of Chicago in 1909 as a graduate student in Physiological Chemistry with Albert P. Mathews. He began investigating the chemical nature and physiological action of the hormone gastrin, a type of biochemical problem that he was concerned with for the remainder of his career. After receiving the Ph.D. degree in 1912, he continued as a member of the faculty, serving as Chairman of the department from 1919, as full professor from 1923, and finally as Distinguished Service Professor of Biochemistry.

He achieved distinction by the guidance and inspiration which he gave to many students; by his contributions to biochemistry; and by his skill in devising equipment and analytical methods. He gave freely of his time and thought in developing the general course in biochemistry for medical students, emphasizing the quantitative and carefully con-

trolled method of experimentation. His method of laboratory teaching is well illustrated by his manual "Practical Methods in Biochemistry." Many graduate students are indebted to him for guidance and stimulation. Sixty of these were rewarded with an advanced degree. With graduate students he practiced the policy of permitting a large degree of independence and encouraged their personal development. His method was that of example rather than precept.

Perhaps his greatest satisfaction was derived from his investigations. He loved laboratory work—the satisfaction of a well planned and executed experiment—the joy of discovery. His enthusiasm and diligence were rewarded by many important contributions to the fields of hormones, vitamins, and analytical methods. Among his most widely acclaimed scientific accomplishments was his demonstration with L. C. McGee of male hormone activity in extracts of bulls' testicles and the development of a method of assay of these extracts with T. F. Gallagher.

Inevitably his distinguished service as a person and a scientist was widely recognized. He was one of this country's delegates to the League of Nations Conference on Standardization of Sex Hormones, an honorary member of the National Medical Society of Buenos Aires; at the invitation of the Uruguayan Government, was this country's representative at the Pan American Congress on Endocrinology; Julius Stieglitz Memorial lecturer; Harvey Society lecturer; one of the Editors of Archives of Biochemistry; President of the Association for the Study of Internal Secretions; a recipient of the Squibb Award by the American Association for the Study of Internal Secretions; recipient of the Annual Award of the Chicago Chapter of the American Institute of Chemists; a member of the Council of the American Society of Biological Chemists, as well as Secretary; several times Vice Chairman of the Chicago Section of the American Chemical Society; and at the time of his death, Vice President. of the Institute of Medicine of Chicago.

It has been frequently stated that distinguished scientists do their most important work before reaching the age of forty. Koch was an exception to this pattern. While he demonstrated capacity and zeal from early manhood, his greatest scientific productivity came after reaching the age of fifty and continued with undiminished vigor until the end. On reaching the retiring age at the University of Chicago in

1941, he transferred his research activities to Armour and Company and two years later became Director of Biochemical Research.

Personally he was modest and unassuming and was very grateful for the confidences placed in him and honors conferred on him. On the occasion of receiving an award he wrote, in a characteristic manner, "I am, of course, very happy over the event not only for myself, but also for my former students, because it really is a recognition of what they have and are doing rather than for what I have done." His seriousness and joy in planning scientific developments at his advanced age and in following the work of others as reported in the journals or at scientific meetings were impressive, and illustrated his abiding faith in the value of the view of an ever expanding scientific interpretation of the universe.

He is survived by his wife, Dr. Elizabeth Miller Koch, also a biochemist, who has been his devoted companion in the laboratory, as well as on many happy vacations in this country and abroad.

That he has lived and worked among us is a cause of deep gratitude to his friends and workers in science.

THOMAS L. MCMEEKIN

The Oxidation of Acetate by Yeast in the Presence of Fluoroacetate

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Received December 15, 1947

INTRODUCTION

Kalnitsky and Barron (1) have reported that maximal inhibition of acetate oxidation by fluoroacetate in yeast was attained only when the inhibitor was added to the cells prior to addition of the substrate. The oxidation was then suppressed almost completely for several hours, but eventually showed a partial "spontaneous release" from the inhibition. The underlying basis of these observations has been clarified by separate investigations in our two laboratories, and is presented in this joint publication.

The initial strong inhibition appears to be due to a fluoroacetate-induced deficiency of essential cellular catalysts such as intermediates of the Krebs tricarboxylic acid cycle. This must be considered a secondary effect of the inhibitor. The true or direct inhibition of acetate oxidation can be determined only when these catalysts are not limiting factors. The latter condition can be most readily achieved by adding inhibitor and substrate simultaneously. If the inhibitor is added first, the cell apparently overcomes the deficiency unaided but only after a long induction period, thus accounting for the "spontaneous release." Ethanol oxidation, in which acetate is an intermediate,[3] is not sensitive to the secondary effect of the inhibitor.

[3] When it is stated that acetate is an intermediate in ethanol oxidation it should be recognized that the true intermediate may be a closely related "active acetate."

EXPERIMENTAL

In both laboratories bakers' yeast (Fleischmann's) was used. In one laboratory (SB) the yeast was starved by shaking a 1% suspension in distilled water under an oxygen atmosphere for 5 hours. In the other laboratory (JOH), 0.05–0.25% suspensions of yeast were made in a solution containing KH_2PO_4 150 mg.-%, $CaCl_2$ 1.0 mg.-%, $MgCl_2$ 1 mg.-%, $FeCl_3$ 0.17 mg.-%, and thiamine hydrochloride 0.01 mg.-% and oxygen bubbled through the medium for 24–72 hours.   All oxygen consumption measurements were made manometrically in Warburg vessels of approximately 15 ml. volume.

The silver[4] and sodium salts of acetyl phosphate were prepared by the methods of Lipmann and Tuttle (2). The purity of the sodium salt was determined from its hydrolysis rate by the method of Lowry and Lopez (3). Succinic semialdehyde was prepared by the method of Carriere (4).

Variations in procedure between the two laboratories which are not noted above are recorded in connection with the individual experiments. Each laboratory was always able to reproduce results obtained in the other in a qualitative way. The quantitative response of the yeast to a given set of conditions, however, usually differed (compare Fig. 1 and Fig. 2).
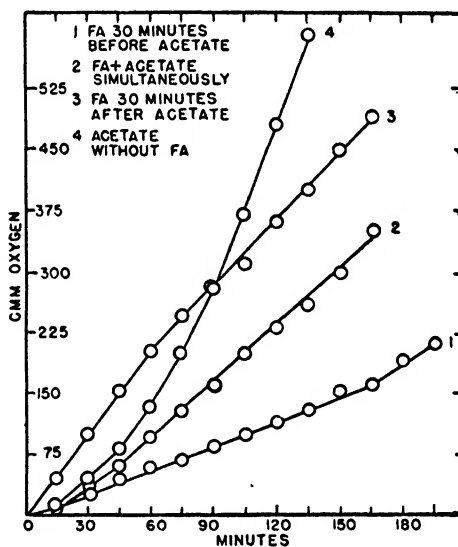
FIG. 1. The effect of order of addition of acetate and fluoroacetate on oxygen consumption of yeast. Yeast (oxygenated in thiamine-salt solution) 18 mg. wet weight; final concentrations: sodium acetate, 10^{-2} M; sodium fluoroacetate (FA), 10^{-3} M. Final volume, 4.4 ml.; pH, 6.0; gas phase, air; temperature, 25°C. Suspending medium as described in experimental section. Zero time indicates time of acetate addition for Curves 1, 2, and 4, and time of FA addition for Curve 3.

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[4] The silver salt was prepared by Dr. Harold Persky.

I. The Effect of Time of Addition of Inhibitor with Respect to Substrate

In Fig. 1 data are plotted showing the stimulation of oxygen consumption by acetate when added to the yeast at different times with respect to fluoroacetate. When the two were added together the rate became linear after about 15 minutes. When acetate was added first about one hour was needed to attain linearity, and when fluoroacetate
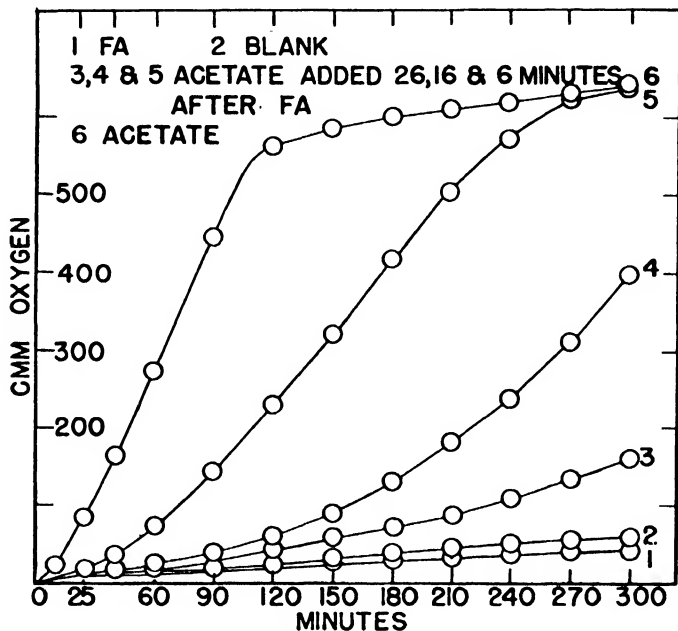
FIG. 2. The induction period in oxygen consumption of fluoroacetate (FA)-treated yeast in the presence of acetate. Final concentrations: sodium acetate, 10^{-2} M; sodium fluoroacetate, 10^{-3} M. All other conditions as described for Table I. Zero time indicates time of acetate addition in all cases.

was added 30 minutes before acetate nearly 3 hours elapsed before a constant rate resulted. The final rate in all cases was the same. These data indicate that simultaneous addition of the two substances leads to most rapid attainment of a steady oxidation rate and is the most satisfactory manner of studying the effects of this inhibitor.

Several factors must be considered in interpreting these curves. In the case of prior addition of acetate, oxidation was already very active at the time of inhibitor addition. The time required to achieve the final

steady rate must in this case represent the equilibration time of fluoroacetate with the cell. When the two are added simultaneously at least two antagonistic effects are expected to be operative: (1) a steadily increasing oxygen consumption rate as evidenced in the normal control curve, and (2) a developing inhibition as fluoroacetate slowly penetrates into the cell. The rate of oxygen uptake at any given time should thus have been influenced by both of these factors. In the case of prior addition of inhibitor a long induction period in oxygen consumption resulted which, because of its duration, and the rapidity of increase in oxygen uptake in the control cannot be explained on the basis of equilibration time with respect to acetate but rather seems to be an extension of the normal induction period.

Fig. 2 shows the results of an experiment similar to that of Fig. 1, with yeast given different pre-experimental treatment. The length of the induction period was dependent on the length of the interval between additions of inhibitor and substrate. This experiment bears a striking similarity to one performed by Lynen (5) in which the duration of the induction period in acetate oxidation in starved yeast was related to the time of oxygenation prior to addition of substrate.

On the basis of experiments reported below, this fluoroacetate-induced induction period may be considered due to a deficiency in the poisoned cell of certain substances which are essential for acetate oxidation in yeast (5). Such a deficiency could arise in the initial concentrations of these substances were low and the poison inhibited their formation. An alternative hypothesis is that the inhibitor blocks some step in the endogenous respiration causing an exhaustion, by oxidation, of the essential compounds initially present. It is not possible on the basis of available data to choose between these possibilities.

II. Reversal of Inhibition Due to Prior Addition of Fluoroacetate

If the hypothesis of a deficiency in essential catalysts is the correct interpretation of the strong initial inhibition, or induction period, produced when fluoroacetate is added to the yeast before acetate, this inhibition should be released by such substances if they are capable of penetrating the cell, or by compounds capable of yielding them after entering the cell. Data are presented in Table I which show that succinate and ethanol were capable of producing such an effect, and that each potentiated the action of the other. Acetaldehyde (Table II)

TABLE I

The Effect of Succinate and Ethanol on the Oxygen Consumption of Fluoroacetate-Treated Yeast in the Presence of Acetate

Yeast (oxygenated 5 hours in distilled water), 4 mg. dry weight; sodium fluoro-acetate, 10^{-3} M added to yeast in main chamber 26 minutes before tipping in acetate; KH_2PO_4, 2×10^{-2} M; final volume, 2 cc.; incubation volume, 1.8 cc.; gas phase, air; temperature, 28°C.; center well contained 0.15 cc. 15% KOH; duration of experiment, 3 hours. Substrate concentrations: acetate, 10^{-2} M; ethanol, 10^{-3} M; succinate, 0.1 M. Acetate and ethanol were added from the side arm; succinate was added to the main chamber of the Warburg vessel. The pH of all added solutions was adjusted to 4.8. All concentrations given are final values.

Substrates	Oxygen uptake		
	Total	Due to ethanol	Due to succinate
	mm^3	mm^3	mm^3
None	26		
Acetate	39		
Succinate	37		11
Acetate + succinate	81		42
Ethanol	110	84	
Ethanol + succinate	119	82	9
Acetate + ethanol	183	144	
Acetate + ethanol + succinate	312	231	129
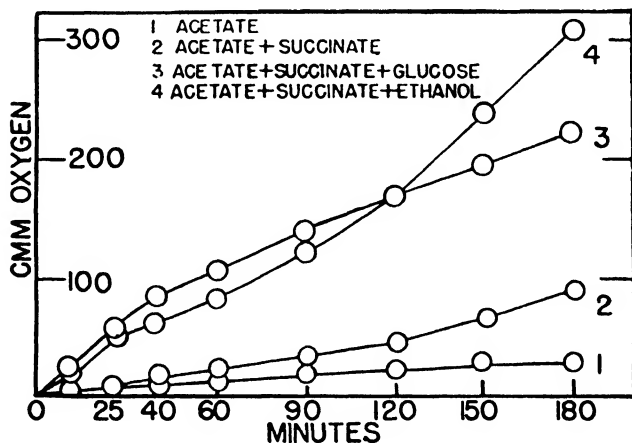
FIG. 3. The effect of succinate, glucose, and ethanol on the oxygen consumption of fluoroacetate (FA)-treated yeast in the presence of acetate. Glucose final concentration, 10^{-3} M. All other conditions as described for Table I.

TABLE II

The Effect of Several Added Substrates on the Oxygen Uptake of Fluoroacetate-Treated Yeast in the Presence of Acetate and Succinate

Conditions as in Table I, except that all vessels contained 0.1 M sodium succinate buffer, pH 4.8. All other substrates were added from the side arm of the vessel.

| | Oxygen uptake | |
| | Total mm.³ | Due to substrate other than acetate mm.³ |
Substrates		
None	43	
Acetate, 10^{-2} M	89	
Acetaldehyde, 10^{-3} M	92	49
Acetate, 10^{-2} M + acetaldehyde, 10^{-3} M	340	277
Glucose, 3×10^{-4} M	86	43
Acetate, 10^{-2} M + glucose, 3×10^{-4} M	128	41
None	35	
Acetate, 5×10^{-3} M	86	
n-butanol, 5×10^{-3} M	223	188
n-butanol, 10^{-3} M	74	39
Acetate, 5×10^{-3} M + n-butanol, 5×10^{-3} M	251	165
Acetate, 5×10^{-3} M + n-butanol, 10^{-3} M	125	39
None	54	
Acetate, 10^{-2} M	136	
Acetylphosphate, 1.2×10^{-3} M	75	21
Acetate 10^{-2} M + acetylphosphate, 1.2×10^{-3} M	108	−28

could replace ethanol, but butanol and glucose could not. Acetyl phosphate produced an inhibition.

The evidence for catalytic action is the greater response produced by each compound in the presence of substrate than in the control with no substrate. From Fig. 3 it is apparent that the effect is really a shortening of the induction period. The noncatalytic effect of glucose, compared with ethanol, is apparent from this Figure. Addition of the former substance produced an oxygen uptake curve parallel with the control curve during the latter part of the experiment, in marked contrast to the effect of ethanol.

Succinic semialdehyde (Table III) produced an oxygen consumption by poisoned yeast 4.4 times as large in the presence of acetate as in its absence, compared to a 5.2-fold increase produced by succinate under comparable conditions. This indicates that the two compounds are

TABLE III

*The Effect of Succinic Semialdehyde and Succinate on the Oxygen
Consumption of Fluoroacetate-Treated Yeast in the
Presence and Absence of Acetate*

Yeast, 20 mg. dry weight; final concentrations, sodium acetate 10^{-2} M, sodium succinate 10^{-1} M, succinic semialdehyde 2.5×10^{-3} M, sodium fluoroacetate 10^{-3} M. Fluoroacetate added to yeast 31 minutes before substrates; all substrates added from side arm of vessel; duration of experiment 6 hours; other conditions as described for Table I.

| | Oxygen uptake | |
	Total $mm.^3$	Due to succinate or succinic semialdehyde $mm.^3$
None	85	
Acetate	270	
Succinate	178	93
Succinic semialdehyde	197	112
Acetate + succinate	756	486
Acetate + succinic semialdehyde	764	494

probably equally active on a molar basis, and that the oxidation or presence of the aldehyde group does not produce an additional catalytic action such as the one obtained with acetaldehyde. Because succinic semialdehyde penetrates the cell much faster than succinate,[5] we believe a comparison made on the basis of the oxygen consumption produced by each in control experiments without acetate is superior to one made with equimolar concentrations in the suspending medium, as was done by Lynen (5).

III. The Oxidation of Ethanol in the Presence of Prior Added Fluoroacetate

Because of the catalytic effect of ethanol described above the course of its oxidation in the presence of prior added fluoroacetate is of interest. Kalnitsky and Barron (1) found that when 0.01 M fluoroacetate is added to yeast prior to the addition of ethanol the oxygen uptake stopped when ethanol was oxidized to acetate. From the oxygen consumption data plotted in Fig. 4 it appears that a considerable portion of the acetate, which presumably is formed as a product of ethanol oxidation, was oxidized under our conditions (10^{-3} M fluoroacetate).

[5] This conclusion is inescapable on the basis of Lynen's experiments (5, 6).

Calculations from the plotted data show that, with ethanol concentrations of $10^{-3} M$, $3 \times 10^{-3} M$, and $10^{-2} M$ oxygen, equivalent to 61, 47, and 31%, respectively, of the theoretical maximum of formed acetate, was absorbed in 3 hours, in addition to the oxygen required for oxidation of alcohol to acetate. This corresponds to 1.2, 2.8, and 6.2 μM of acetate. The oxygen consumption in a comparable experiment with $10^{-2} M$ acetate (Table I) was equivalent to 0.3 μM. This amounts to 1/4 that found with ethanol at 1/10 the molar concentration, or to 1/20 that obtained with an equivalent concentration of ethanol.
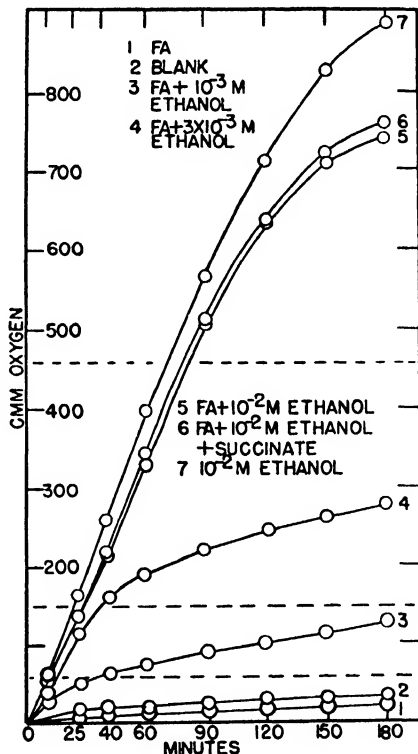
FIG. 4. The oxygen consumption of fluoroacetate-treated yeast in the presence of ethanol. Sodium fluoroacetate (FA), $10^{-3} M$ final concentration, added 27 minutes before tipping in substrate. Sodium succinate buffer, pH 4.8, 0.1 M final concentration, added to main chamber of vessel. Other conditions as described for Table I. Broken lines represent the theoretical oxygen uptake for complete conversion of ethanol to acetate for the three ethanol concentrations.

Oxidation of the acetate formed from ethanol under these conditions apparently proceeds with no induction period, such as is evident in the

oxidation of added acetate. If there was such an induction period in this case it should be indicated by an inflection in the oxygen uptake curve at the point where sufficient oxygen was consumed to convert all of the ethanol to acetate. This was shown to be true in experiments by Lynen (6) in which small quantities of ethanol did not completely overcome the long induction period in the oxidation of acetate by exhaustively starved yeast.

According to Lynen (5, 6) acetate and ethanol are oxidized *via* the Krebs cycle in yeast, and succinate serves as a source of oxaloacetate for the initial condensation reaction. In our experiments, although succinate catalyzed the oxidation of acetate, it had no significant effect on the oxidation of ethanol. This fact is hard to explain satisfactorily. It may be due, as Lynen, who noted a similar anomaly in some of his experiments, suggests, to a coupling of the oxidation of ethanol to acetate with formation of oxaloacetate from pyruvate and CO_2.

IV. The Effect of pH and of Inhibitor Concentration

Many weak acids and bases appear to penetrate the surface of living cells only in the form of undissociated molecules (7, 8). The concentration of material in the cell interior is thus determined as a function of undissociated molecules. extracellularly, which, in turn, is determined by pH. Also, the time required to achieve a given concentration intracellularly depends on this factor. For a substance such as fluoroacetic acid ($pK_a = 3.81$) a change in pH from 6.0 to 4.0 increases the concentration of undissociated fluoroacetic acid molecules 60-fold.

In Table IV are given oxygen consumption data for yeast cells to which a low concentration of fluoroacetate had been added prior to addition of acetate. No account was taken of the final equilibrium state in this experiment, but the data nevertheless indicate the great importance of pH control in studies of this kind which involve living cells and an acidic inhibitor as well as substrate. As may be seen from the table, as the pH was lowered the oxygen consumption of the poisoned cells, compared to controls at the same pH value, dropped from 97 to 16%.

In Fig. 5 are shown the results of simultaneous addition of inhibitor and substrate at two pH values and with two inhibitor concentrations. The final rate of oxygen consumption was independent of pH in these experiments. The equilibrium condition was not attained as rapidly

TABLE IV [a]

The Effect of pH on Inhibition of the Oxygen Consumption of Fluoroacetate-Treated Yeast in the Presence of Acetate

Yeast (oxygenated 5 hours in distilled water), 10 mg. dry weight; sodium fluoroacetate, 2×10^{-6} M; acetic acid, 0.01 M brought to the same pH value as that of the buffer. Buffers, hippurate, and hippurate-phosphate, 0.028 M. Blank value subtracted in all cases. Oxygen uptake of yeast with no substrate was from 24 mm.[3] at pH 3.8 to 35 mm.[3] at pH 6.2. Duration of experiment, 1 hour.   Temperature 27°C. FA added 25 minutes before acetate addition.

	Oxygen uptake		
pH	Acetate	Acetate + FA	Acetate + FA per cent of acetate control value
	mm.[3]	mm.[3]	
3.80	280	46	16
4.77	296	105	33
5.20	352.4	181.7	52
6.24	311.4	302.6	97

[a] These data were provided by Prof. E. S. Guzman Barron.

at the lower pH value, however, apparently because of the shorter normal induction period in the control, which in turn must be due to more rapid acetate penetration.

In Fig. 5 is illustrated, in addition to the pH effect, the increased inhibition obtained, as evidenced in the final equilibrium condition, by increasing the inhibitor concentration. It should be pointed out that the effective concentration of inhibitor, determined with living cells, is of little value in approximating the effective concentration at its ultimate site of action, *e.g.*, about a particular enzyme, without additional information concerning its distribution between the interior and exterior of the cell and within the cell. Work with cell-free preparations of animal tissues (10, 11) has shown effective concentrations, in general, to be much higher than those used by us with yeast cells.

Discussion

Time of Addition of Inhibitor

The significance of the effect of order of addition of inhibitor and substrate is especially apparent when an attempt is made to determine the quantitative effectiveness of the inhibitor in the presence of acetate

or other substates. For example, if values for per cent inhibition are
calculated from the data plotted in Fig. 1, using total oxygen consump-
tion values at some arbitrary time, such as 2 hours, three different
results will be obtained. However, if inhibition is calculated in terms of
the rate of oxidation in the final equilibrium state compared with the
control rate, uniform and reproducible values result. One of us (9)
has found, using the final rate criterion, that pyruvate oxidation is
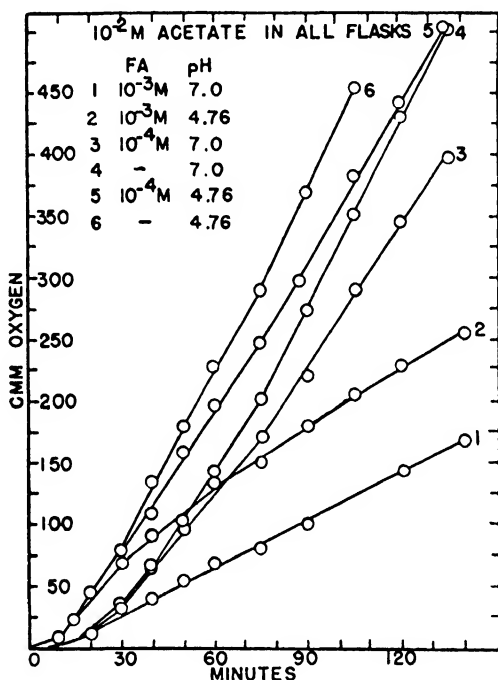
FIG. 5. The effect of pII on inhibition by fluoroacetate of oxygen consumption of
yeast in presence of acetate. Conditions as described for Fig. 1.

much more sensitive to fluoroacetate inhibition than acetate oxidation.
This contradicts the resport of Kalnitsky and Barron (1) that acetate
oxidation is more sensitive. The contradiction is readily resolved when
it is considered that the latter workers used oxygen consumption values
obtained during the fluoroacetate-produced induction period to
calculate per cent inhibition, and that the induction period in pyruvate
oxidation is much shorter than the corresponding period for acetate (9).

The Succinate and Ethanol Effects

A general interpretation of the autocatalytic nature of the oxidation of acetate by fluoroacetate-poisoned yeast involves the assumption that an essential step in the oxidative pathway is catalyzed by the presence of one or more of the intermediates in the overall process The poison, in the absence of substrate, causes an exhaustion of these substances. Subsequent addition of acetate allows their slow accumulation either from the endogenous metabolism of the cell or from the substrate, and the rate of oxidation gradually increases in consequence. Thus, as previously mentioned, succinate accelerates the autocatalytic process by serving as a source of oxaloacetate, essential in the initial reaction of the Krebs cycle. The effect is catalytic in nature because the intermediate is continually regenerated *via* the cycle.

That oxaloacetate is not the sole limiting catalyst in the poisoned cells is clearly evident from the fact that the succinate effect is strongly potentiated by ethanol or acetaldehyde. According to Lynen the oxidation of acetaldehyde supplies energy for the initial condensation reaction of the Krebs cycle, and succinic semialdehyde oxidation serves this purpose once the cycle is activated. Our results with succinic semialdehyde, in which it was not more active as a catalyst than succinate, do not confirm this view. The theory may apply, however, if some intermediate other than succinic semialdehyde, preceding succinate in the cycle, is assigned this function. α-Ketoglutarate, for example, may activate the condensation, especially in view of its specific action in promoting citrate synthesis from acetoacetate (12). Unfortunately, this compound, as well as citrate, is unsuitable for testing under our experimental conditions.

No definite conclusions can be drawn from our experiments concerning the possible role in ethanol or acetate oxidation of acetyl phosphate, for reactions involving loss of phosphorus prior to oxidation were not eliminated. It would be difficult to do this with living cells.

SUMMARY

1. The time of addition of fluoroacetate to yeast with respect to acetate effected the initial rate of oxygen consumption. After an extended period, the rates became constant and identical regardless of the relative order of addition.

2. When fluoroacetate was added to yeast 25–30 minutes prior to acetate a significant oxygen uptake did not occur until an extended induction period had elapsed. Oxidation during this induction period was catalytically stimulated by ethanol, acetaldehyde, succinate, and succinic semialdehyde. Ethanol and succinate each potentiated the effect of the other.

3. Acetate, which presumably is formed as an intermediate in ethanol oxidation, was oxidized at a good rate in fluoroacetate poisioned yeast under conditions very unfavorable to the oxidation of added acetate.

4. Some effects of varying pH values on the effectiveness of fluoroacetate as an inhibitor of acetate oxidation in yeast cells are described.

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Ethylene as a Metabolic Product of the Pathogenic Fungus, *Blastomyces dermatitidis*

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Received September 10, 1947

INTRODUCTION

It has been demonstrated repeatedly (2, 10, 11, 16, 17) that the evolution of ethylene as a gaseous metabolic product of ripening fruit in particular, and plant tissues generally, can be detected by certain highly specific biological tests—epinasty of tomato and potato leaves, the "triple" response of etiolated pea seedlings, inhibition of seed germination, and the acceleration of fruit ripening. Pratt and Biale (15) state that this is now so well established and generally accepted that demonstration of the activity of an emanation by any of the above methods is a very strong indication that ethylene is among the gases evolved.

Thus far only one fungus, *Penicillium digitatum*, has been shown to produce ethylene (2, 11). This fungus is among those most commonly isolated from decaying fruit.[2]

The oxidative metabolism of *Blastomyces dermatitidis*, causative agent of North American blastomycosis, or Gilchrist's disease, was first studied by Bernheim (1). In a review of the respiration of pathogenic fungi, Nickerson (12) showed that Bernheim's data implicated the existence of an unsaturated hydrocarbon unless one considered the high endogenous rate of oxygen consumption completely suppressed on the addition of substrate. Subsequent investigations of the respiration of *B. dermatitidis* by Nickerson and Edwards (14) have shown, indeed, that, with acetate as substrate, the endogenous respiration (R.Q. = 1.0) is suppressed, and the oxidation of acetate proceeds with an R.Q. of 1.0 to approximately 80% of completion with the assimilation of 20% of the substrate indicated as (CH₂O).

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[2] Three other fungi commonly isolated from decaying fruit were found (11) to give negative or inconclusive ethylene-production tests. The fungi were: *Diplodia natalensis*, *Diaporthe citri*, and *Alternaria citri*.

Thus, it is clear that any gaseous metabolic product other than CO_2 can be present only in minute quantity.

Since the inference was drawn, as mentioned, that an unsaturated hydrocarbon might be produced by *B. dermatitidis*, experiments were made to see if any such product could be detected.

EXPERIMENTAL

Methods

A. Organisms. Cultures of *Blastomyces dermatitidis*, *B. braziliensis*, and *Histoplasma capsulatum* were obtained from the Duke University Medical School collection through the courtesy of Dr. Norman F. Conant. Stock cultures of these organisms were maintained on Sabouraud's dextrose agar (Difco) and were transferred at intervals of 2 weeks. Experimental cultures were seeded from agar cultures 1 week or less old by transferring a small bit of mycelium.

B. Test for Unsaturated Metabolic Product of Blastomyces. A large scale experiment designed to detect any gaseous metabolic product of *B. dermatitidis* capable of reducing permanganate was first conducted. A 4-liter Erlenmeyer flask containing 3 l. of GGY medium was inoculated with the mycelial form of *B. dermatitidis* and incubated at about 22°C. The medium had the following composition: 2.0 g. glucose, 1.0 g. glycine, 0.1 g. yeast extract-Difco, and 100 ml. distilled water. This flask was arranged so that a current of air was sucked through by means of an aspirator attached to a water tap. The atmospheric air pulled through was passed, in turn, through flasks of water, ethylene glycol, water, the culture, water, 2% potassium permanganate, and water. Preliminary checks showed no reduction of the permanganate by atmospheric air when passed through the ethylene glycol trap and washed with water before entering the permanganate. This culture was maintained for 30 days, at the end of which time the fungus culture had attained a considerable bulk and discoloration of the permanganate was evident. The permanganate was then titrated with oxalate.

C. Biological Tests for Ethylene. The "triple response" of etiolated pea seedlings to very small concentrations of ethylene is marked. The response is characterized (7) as a "change of negative geotropism to diageotropism, increased growth in thickness, and reduced rate of growth in length." The test procedures employed were essentially those of Pratt and Biale (15). Alaska peas were soaked 7 hours in distilled water, then placed on wet filter paper in Petri dishes, 50 seeds per dish. After 2 days they were examined and nongerminated or poor-appearing seedlings eliminated to leave about 35 vigorous seedlings per dish (in some instances the selected seedlings were then planted in dishes in sand as given below). Covers of the dishes were then removed, the seedlings were well watered, and placed in a humid atmosphere in the dark for 4 days. At the end of this time, vigorous seedlings about 4 cm. high with good roots were obtained.

Dishes of seedlings were then placed under large bell jars sitting in shallow trays of water (water barrier to gas exchange) in the dark. Cultures of fungi were placed in some of the bell jars along with the peas; other jars had only the peas and served as

controls. In other experiments the pea seedlings were treated with air that had been passed through liquid cultures of *B. dermatitidis* growing in the *mycelial* state (25°C). or in the *yeast* state (37°C.). The fungus cultures were growing in the GGY medium in 1-liter Erlenmeyer flasks maintained in a constant temperature water bath; air was washed, pumped through the culture, then led to the seedlings growing in dishes under a large inverted funnel (covered with black paper) sitting in a tray of water.

For experiments conducted in the light, Alaska peas were planted in dishes 5 cm, in diameter containing washed sand to a depth of 3 cm. Peas were planted 1 cm. deep. 4 to a dish. Two dishes were placed inside each bell jar. A culture of *B. dermatitidis* or *B. braziliensis* in the GGY medium in an Erlenmeyer flask was also placed under the bell jar along with the peas. Controls had peas only under the jars. All bell jars were placed in shallow trays of water. The height of the plants was estimated at intervals to the nearest millimeter without removing the seedlings from under the bell jars. Plants were grown at room temperature (20°–22°C.) with diffuse natural illumination; the light experiments were conducted from Oct. 26 to Dec. 9, 1946.

REDUCTION OF PERMANGANATE BY VOLATILE METABOLIC PRODUCT OF BLASTOMYCES

The permanganate trap through which air had been pulled from the 3-liter culture of *B. dermatitidis* was disconnected after 30 days incubation of this culture at room temperature (20°–22°C.). Ventilation during the experimental period had been at the rate of approximately 1 l./hr. The permanganate was titrated with oxalate, in the usual manner, after being removed from the train; the original titer of the 150 ml. of $KMnO_4$ was 0.568 N; this was found to have been reduced to 0.236 N after the 30 days incubation. It is believed that sufficient precautions were taken in washing the air entering the culture flask to be certain that any reduction of the permanganate resulted from changes in the composition of the air leaving the culture flask. No frothing occurs in aerated *Blastomyces* cultures and, furthermore, precautions were taken to preclude the transfer of droplets from the culture flask. Therefore, it seems safe to conclude that volatile metabolic products of the fungus were responsible for the permanganate reduction.

Assuming that the permanganate was reduced by ethylene alone and that the equation for such reduction may be expressed as:

$$2KMnO_4 + 3CH_2{=}CH_2 + 4H_2O \rightarrow 2MnO_2 + 3CH_2OHCH_2OH + 2KOH,$$
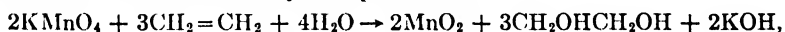
we can calculate that the 1.57 g. of $KMnO_4$ consumed was reduced by 0.42 g. of ethylene. It is doubtful that a quantitative oxidation of ethylene would occur under the conditions used, but the estimate of 420 mg. of ethylene (520 ml. N.T.P.) produced in 30 days by a 3-l. culture gives an indication of the low concentration of the permanganate-reducing substance present in the volatile metabolic products.

RESPONSE OF ETIOLATED PEA SEEDLINGS TO GASEOUS PRODUCTS OF BLASTOMYCES

It is evident, since there was no direct contact between the cultures of fungi and the pea seedlings, that any differences observed between

the control seedlings and those grown in the presence of the fungus must be caused by changes in the gaseous atmosphere. As can be seen in Fig. 1, the triple response, characteristically brought about by ethylene, is markedly shown by pea seedlings grown in the presence of liquid cultures of *Blastomyces dermatitidis*. Evidently this fungus produces ethylene in appreciable quantity, if one accepts the pea
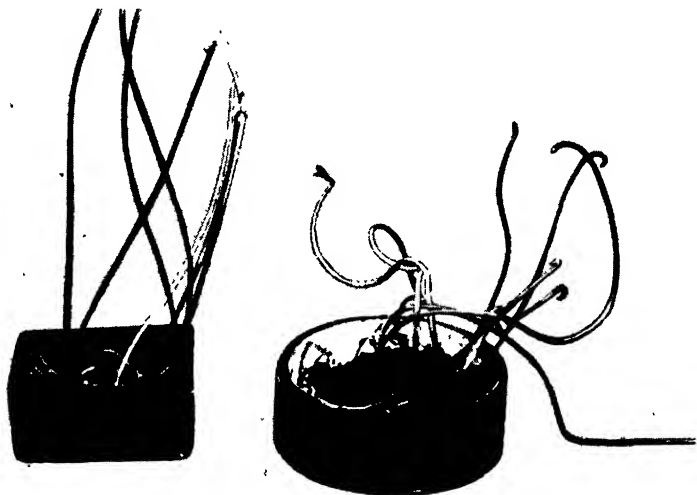
FIG. 1. Growth of Alaska pea seedlings in the dark; control (left), and in presence of culture of *Blastomyces dermatitidis* (right). Seedlings grown for 10 days in the dark under bell jars. Note the pronounced diageotropism of stems of seedlings at right.

seedling response test as specific proof of the presence of ethylene. The seedlings shown in Fig. 1 were allowed to grow for 10 days under the bell jars. Actually, the test period need not be this long; marked differences, with a characteristic ethylene reaction, may be observed between control and experimental plants within a 4-day test period, provided vigorous cultures of *Blastomyces* are employed.

Measurements were also made on the effect of the presence of the

fungi on peas germinated and grown in the light. As shown in Fig. 2 the presence of cultures of *B. dermatitidis* or *B. braziliensis* caused a significant decrease in the height attained by the seedlings. Marked shortening and thickening of the pea seedlings caused by the presence of the fungi was most noticeable in experiments conducted in the light;
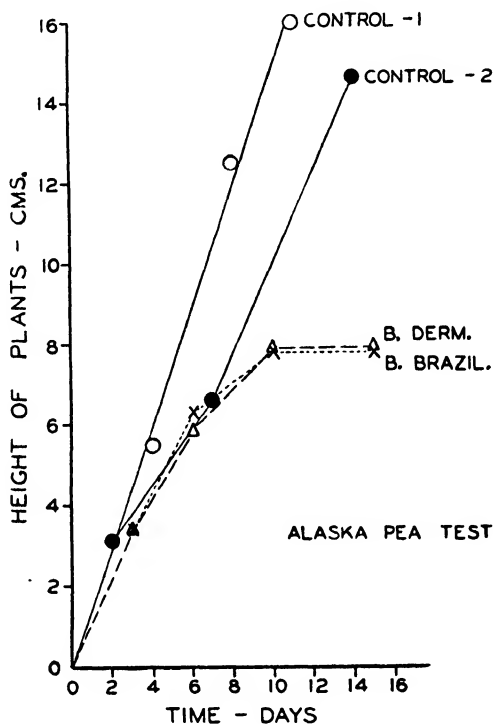
FIG. 2. Graph of results of Alaska pea test for ethylene production by *Blastomyces dermatitidis* and *B. braziliensis*. The points on control curve 1 represent the averages from 8 plants, those of control 2 from 4 plants. In the experimental figures each point represents the average of measurements on 12 plants. (Experiments by W. J. N. & C. George.)

more so than in the experiments conducted in the dark. This effect has been observed by others working with the "triple response" test. For the tests conducted in the light, both agar (Difco-Sabouraud's agar) and liquid cultures (50 ml. GGY medium in 125 ml. Erlenmeyer flasks) of the fungi in the mycelial state were employed (see Nickerson and

Williams (13) for discussion of the phenomenon of thermal dimorphism in these 3 pathogenic fungi). Ethylene effects were obtained from both solid and liquid cultures of both species of *Blastomyces*.

Gaseous metabolic products of the fungi had a marked inhibitory effect on the germination of Alaska peas (first soaked 7 hours in water), as can be seen in Fig. 3. In these experiments the dishes with

FIG. 3. Seeds of Alaska peas germinated for 2 days in Petri dishes, then subjected to different treatments. (a) 1 p.p.m. ethylene; (b) aeration from *Histoplasma capsulatum* culture in mycelial stage for 4 days; (c) aeration from *Blastomyces dermatitidis* culture in yeast stage for 4 days; (d) same as "c" but received vapors for only 1 day, then removed to dark, humid cabinet—no further treatment; (e) duplicate of "c."

peas were placed under inverted funnels (in the dark) and aerated with air previously passed through liquid cultures (GGY medium) held at 25°C. or 37°C. (*B. dermatitidis*), and 25°C. (*Histoplasma capsulatum*). Fig. 3a shows for comparison a lot of seedlings that had germinated in the presence of a known concentration (1 p.p.m.) of ethylene. One sample of seeds was exposed for only 24 hours, then removed from the presence of the vapors; the 24 hour exposure (Fig. 3d) was sufficient to affect markedly the germination of the seeds.

Discussion

On the basis of the experiments reported it is highly probable, though it has not been conclusively proved, that ethylene is a normal metabolic product of *Blastomyces dermatitidis*, *B. braziliensis*, and at least the mycelial stage of *Histoplasma capsulatum*. The amounts of ethylene produced are minute, amounting to but a small fraction of the carbon metabolized by the fungi when an exogenous substrate is available.

Since the pathogenesis of blastomycosis and of histoplasmosis is largely unexplained, a question might be raised as to the participation of ethylene in these pathogenic processes. The probable production of ethylene by these fungi has been here demonstrated only *in vitro*, yet a few speculations may be warranted in light of the known *in vivo* action of ethylene on ripening fruit!

The principal effect of ethylene on green orange rinds is to stimulate degreening (9); carotenoids in the peel show no significant change as a result of ethylene treatment (8, 9). A chlorophyll-decomposing enzyme has been demonstrated as the natural cause of degreening of rinds of the satsuma orange (5). The action of ethylene in fruit ripening has been concluded to be a stimulation of chlorophyll decomposition (3, 9). The mechanism of this action is unknown, although Lynch (8) has postulated a coenzyme action for ethylene in the process. In view of the structural relationships between chlorophyll and hemoglobin it seems very suggestive that a hypochromic anemia is known to be involved in blastomycosis and in histoplasmosis (4).

No information is available on the effect of long continued application of ethylene to lung tissue. High concentrations over a short period have an anaesthetic action (6). An explanation of the localized caseation phenomena in blastomycosis and in tuberculosis has been sought, but is not to be explained on the basis of toxin production or by

mechanical action of the causative organisms. The similarities clinically between the diseases blastomycosis and tuberculosis and between the causative organisms (high wax and fat content, particularly) would seem to indicate that it is worthwhile to examine this working hypothesis experimentally. Experiments are in progress to determine whether the *Mycobacteria* produce an ethylene-positive test reaction. Experiments are also in progress to furnish more conclusive proof on the production of ethylene by the fungi herein discussed.

SUMMARY

It has been demonstrated that a gaseous metabolic product of *Blastomyces dermatitidis* is capable of reducing a solution of potassium permanganate. On the assumption that the product is ethylene, it is calculated that about 420 mg. are produced from a 3-liter liquid culture in 30 days.

Employing the "triple response" reaction of Alaska pea seedlings, generally agreed to be a specific biological test for small concentrations of ethylene, it has been shown that gaseous metabolic products of *B. dermatitidis* cause a typical ethylene reaction in the pea seedlings.

Gaseous metabolic products of *B. dermatitidis* and *B. braziliensis* are shown to inhibit the growth of pea seedlings in the light (ethylene-like reaction). Similar products of the yeast and mycelial stages of *B. dermatitidis*, and of the mycelial stage of *Histoplasma capsulatum*, inhibit to a high degree the germination of Alaska peas and cause a diageotropism in the seedlings that succeed in germinating (ethylene-like reaction).

From the known action of ethylene in stimulating chlorophyll decomposition in the ripening of fruit, some speculations are presented as to a possible role of ethylene in the pathogenesis of blastomycosis and histoplasmosis.

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Characterization of Tomato Pectinesterase

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INTRODUCTION

Pectinesterase is present in practically every type of plant tissue (1), particularly in the members of the *Solanaceae* (2). However, few sources contain it in abundance except tomato fruit (3) and citrus peel (4). Earlier work on the enzyme is mainly due to Kertesz and co-workers (3, 5, 6, 7), MacDonnell *et al.* (4), Lineweaver and Ballou (8), and Holden (2). The present work on tomato pectinesterase was completed before the recent publications on the subject by McColloch and Kertesz (9) and by Hills and Mottern (10) came to the notice of the authors. This paper, therefore, reports the results of these investigations only where they serve to bring out new observations or emphasize quantitative differences in the properties of the enzyme.

EXPERIMENTAL

Measurement of Activity

Enzyme activity was followed by a modified colorimetric determination of the methanol liberated from pectin solution (11, 12). The experimental details of the method adopted were essentially as detailed by Holden (2, 13), except that the whole of the reaction mixture at the end of 10 minutes' deesterification was distilled for methanol determination instead of a 5 cc. sample. A purified preparation of citrus pectin (moisture, 8.6%; ash, 1.15%; methyl ester, 4.18%) was used as substrate. An 0.8% solution of this pectin had a pH of 3.6 and was always neutralized before use as substrate or in buffer-substrate mixtures. Color measurements were effected in a Klett-Summerson photoelectric colorimeter with blue filter in position; methanol concentrations were arrived at by use of a reference curve with known amounts of purified methanol (14).

Enzyme activities were measured at 27°C. and expressed in pectinesterase units (2). One unit of enzyme represents that amount which would liberate 32 mg. of methanol from pectin in 1 min. in $M/10$ Na$_2$HPO$_4$ solution at pH 8 and at 27°C. ($\pm 1°$).

Distribution of Activity

As observed by Kertesz (5), pectinesterase activity in the tomato increased rapidly with ripening, and was associated mainly with the pulp (7, 15). The distribution of the enzyme in the juice, the serum obtained by centrifuging, and the pulp after extraction with 1:10 $M/10$ Na$_2$HPÓ$_4$ at pH 8 are given in Table I.

TABLE I
Location of Pectinesterase Activity

	Activity units/g. of				Per cent of total activity		
	Fruit	Pulp	Juice	Serum	Pulp	Juice	Serum
Raw fruit	0.1773	0.145	0.00532	0.000823	72.7	27.3	4.2
Ripe fruit	0.2377	0.334	0.00935	0.00349	62.4	37.6	13.4

The activity in the juice is evidently due to the suspended cell debris removable by centrifugation. Approximately 87% of the total activity in ripe fruit may be stated to exist in the pulp.

Extraction

Salt concentration and pH are important factors determining the efficiency of enzyme extraction (4, 2) and, only over a limited range of pH, pectinesterase is desorbed from pulpy plant tissues (15). Extraction of enzyme by adjusting the pulp to slightly alkaline pH with NaOH solution has been recommended on this basis (16, 7). In Table II are given the results of a quantitative comparison of the activity of 10% extracts of minced pulp, obtained after squeezing by hand in extractants adjusted to different pH values, the extractions being carried out by soaking for one hour.

The optimum pH for extraction would be 8; at this pH, phosphate buffer is better than salt solution as it minimizes any pH drift. Water alone maintained at pH 8 extracts less of the enzyme than phosphate solution, presumably because of salt concentration.

The activities of 3 successive extracts obtained by soaking the pulp each time for one hour with phosphate solution at pH 8 were, respectively, 81.2, 14.9, and 3.9% of the total. Longer periods of soaking than one hour gave darker extracts containing contaminants with but little increase in total activity. For the following experiments, therefore, the extract, as prepared by soaking the minced pulp for one hour in 1:10 wt./vol. of 0.1 M Na$_2$HPO$_4$ at pH 8, followed by centrifuging at 3000 r.p.m. for 15 minutes, was used. It had an activity of 0.0375 units/cc. and could keep well for fairly long periods when stored in the ice chest.

TABLE II

Relative Merits of Different Extractants

Extracting reagent	pH of extractant	pH of extract	Activity units/ cc. of extract	Relative activity
				Per cent
Water	7.5	4.1	0.00128	4.9
Water[a]	8.0	8.0	0.02280	87.0
NaCl (0.885 M)	6.0	4.1	0.0150	57.2
NaCl (0.885 M)	8.0	4.1	0.0225	85.9
Na₂HPO₄ (0.1 M)	6.0	6.0	0.0172	65.6
Na₂HPO₄ (0.1 M)	7.0	7.0	0.0193	73.6
Na₂HPO₄ (0.1 M)	8.0	8.0	0.0262	100.0

[a] pH maintained by addition of $N/10$ sodium hydroxide every two minutes.

PARTIAL PURIFICATION OF THE ENZYME

To a known volume (100 cc.) of the pectinesterase extract at pH 8, ammonium sulfate was added to reach 10% concentration. The extract was then centrifuged and the precipitate obtained dissolved in 25 cc. of phosphate solution buffered at pH 8 and its activity measured. To the supernatant liquid obtained after centrifuging, more ammonium sulfate was added to reach varying strengths from 20 to 60% concentration and the activity determined as above on each of the fractions obtained as precipitate. The results are shown in Fig. 1. For comparison, the results obtained by fractional precipitation with alcohol are included.

Ethanol precipitation of the enzyme from phosphate extracts resulted in practically complete loss of activity; precipitations in the cold were not attempted (*cf.* 8, 17, 18). Addition of ammonium sulfate did not appreciably lower the pH of the phosphate solution, because of the latter's buffering capacity; the maximum pH change was from 8 to 6.0 which was well within the range for activity of the enzyme. The results, included in Fig. 1, showed that, at between 30 and 50% concentration of ammonium sulfate, most of the enzyme is precipitated, while, at 10 or 20% concentration, although the precipitate obtained was comparatively more, it had very low activity. The procedure finally adopted for obtaining an enzyme concentrate was therefore as follows:

To 100 cc. of the phosphate extract of the pulp at pH 8, ammonium sulfate was added to a concentration of 20% and the precipitate which resulted was discarded after centrifuging. The supernatant was treated with more ammonium sulfate to a concentration of 50%, the precipitate separated by centrifugation, and dissolved in 25 cc. of Na₂HPO₄ solution at pH 8. This extract had about 75% of the activity in the original solution and was further purified by a second salting-out with ammonium sulfate in the same manner as above, when a preparation was obtained retaining 60% of the enzyme originally present and with an activity of 0.56 units/mg. dry wt. as compared with 0.0019 units in the original extract and representing, therefore, a 300-fold concentration of the enzyme. The enzyme preparation obtained as above could be dried *in vacuo* at room temperature without any loss in activity, the yield being about 4 mg./100 cc. of the original extract.

McColloch *et al.* (7) had adopted a procedure of purification by dialysis of tomato pectinesterase extracts in sodium chloride solution. It was, however, observed in a study of the comparative merits of the two methods that single dialysis as recommended by McColloch *et al.* (7) resulted in nearly 40% loss of the enzyme initially present while a second dialysis yielded a product with only 40% of the initial activity.
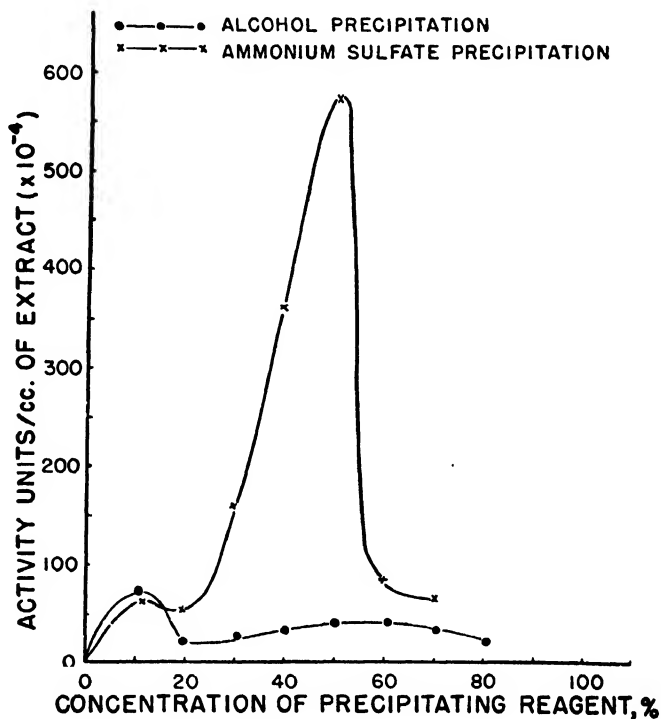
FIG. 1. Fractional precipitation of the enzyme.

The dialysis method was also a slow one as compared to fractional precipitation with ammonium sulfate. In the latter case, the only precaution of importance was to carry out the precipitation at a pH not very much below 7. The buffering capacity of the phosphate in the extract sufficed to maintain the pH above the inactivation limit.

PROPERTIES OF THE ENZYME

Variation of Activity with pH

Variations in activity with pH were studied by adding 0.5 cc. of dialyzed and diluted (six times) enzyme extract to buffer substrate mixture containing 5 cc. of pectin solution and 5 cc. of buffer of re-

quired pII. Corrections were made for nonenzymic demethylation by carrying out tests without addition of enzyme. The activity was maximum at pII 7.8, which agrees closely with the reported values (9, 10, 19).

Variation of Activity with Time

The rate of deesterification was followed with 0.2 cc. of 1:6 diluted enzyme allowed to act for varying periods up to 30 minutes on the buffer substrate mixture; reaction was stopped at stated intervals by addition of acid. The results showed that, during the first 20 minutes, the activity bore a linear relationship with time. In this time, 45% of the methanol had been split off.

Variation of Activity with Enzyme Concentration

One-tenth to 1.0 cc. of pectinesterase extracts (1×10) were added to various lots of buffer substrates at pII 8.0 and the changes in activity measured as usual. Activity increased with enzyme concentration, the relationship being linear except at high concentrations of enzyme when it was observed that there was extensive demethylation as shown by gel formation on addition of acid to stop enzyme action.

Variation of Activity with Substrate Concentration

The relationship subsisting between substrate concentration (0.1–1.0% pectin) and activity was generally in agreement with earlier observations (3, 10) and, as pointed out by Kertesz (3), the optimum concentration of substrate for enzyme activity varied slightly with different pectin preparations; with one preparation, however, there was a steady decrease in rate of demethylation beyond 0.3–0.4% concentration. This behavior was inexplicable. Whether it may be due to any inhibiting factor operating at high concentrations of the pectin is being studied further.

Variation of Activity with Temperature

The tests were carried out by keeping for about 5 minutes buffer substrate mixtures (pII 8.0) in a water bath thermostatically main-

tained at various temperatures and then adding 0.2 cc. of enzyme extract (1:4 dilution). Controls without added enzyme were simultaneously carried out under identical conditions for nonenzymic demethylation which was appreciable. Fig. 2 gives the results which show that the optimum temperature for pectinesterase activity is about 50°C. for 10 minutes' hydrolysis. This value is low in comparison with the
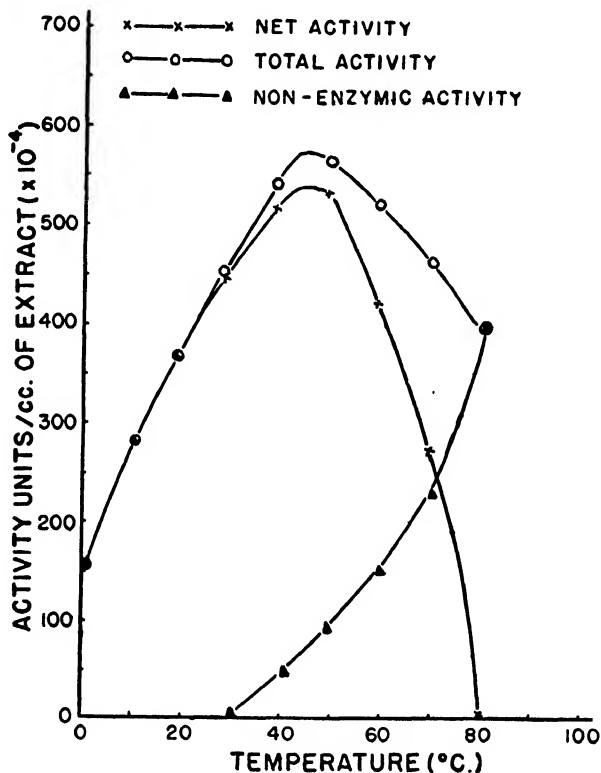
FIG. 2. Variation of activity with temperature.

values recently reported (9, 10); obviously, reaction time and pH are factors influencing the optimum temperature for deesterification. Besides, as seen from Fig. 2, at higher temperatures thermal degradation of pectin is quite appreciable at pH 8, whereas at 6.0–6.5, which was the pH used by the earlier workers, nonenzymic demethylation would obviously be much less.

Stability of the Enzyme

Effect of pH. The dialyzed enzyme solution was diluted 10 times with different buffers so that the resultant mixtures had pH values varying from 2 to 9; these were kept both at 0°C. and at room temperature for one week. Their activities were determined thereafter at pH 8 as usual, using 1 cc. lots of the enzyme solutions. The results are shown in Fig. 3. The enzyme is fairly stable in the region of its optimum pH, *i.e.*, 6–8. According to Hills and Mottern (10), tomato pectinesterase is most
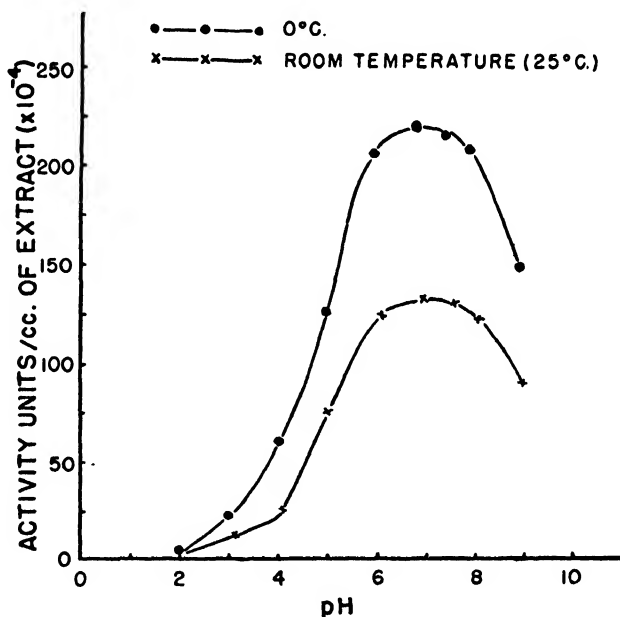
Fig. 3. Stability of enzyme with pH.

stable at pH 4, the natural pH of tomato tissue, but the loss in activity at higher pH is not much. It was observed that there was some precipitation below pH 5 and though the precipitates were associated with some enzyme activity, activity measurements were carried out for the supernatants only. The actual loss in activity at pH 4 may not, therefore, be as much as is indicated by the results.

Effect of Temperature. The effect of temperature on the stability of the enzyme extract at pH 8 was determined by adding the enzyme

preparations (1:10 dilution) at pH 8 to empty tubes previously brought to various temperatures in a water-bath and keeping at these temperatures for 5 minutes. They were immediately cooled and the activities determined by the routine procedure. The results (Fig. 4) show that at 80°C. there is complete inactivation of the enzyme, while at 70°C. it retains only 4.4% of its activity. At 80°C., the loss in activity after exposure for 15 and 30 seconds was 45.5 and 97.7%, respectively. As
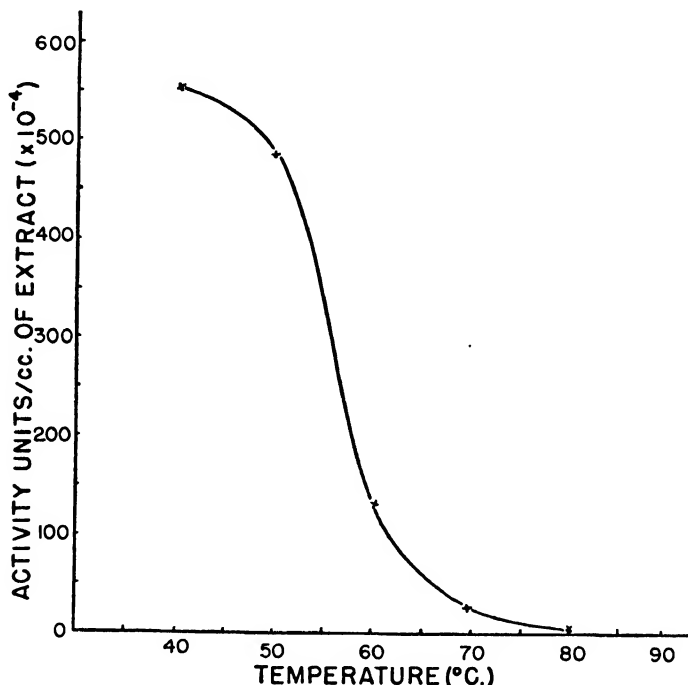
FIG. 4. Stability of enzyme with temperature.

observed by Kertész (6), the enzyme was completely destroyed at this temperature in 45 seconds.

Effect of Dialysis. Phosphate extract of the enzyme at pH 8 was fairly stable, retaining its activity for a considerable period when stored at 0°C.; on the other hand, a dialyzed extract of the enzyme had lost nearly half its activity within one month when stored at 0°C. The fall in activity was rapid at the outset and was only gradual thereafter

(Fig. 5). At room temperature, the dialyzed solution had lost all its activity within 15 days. These results are similar to those of MacDonnell *et al.* for orange pectinesterase and suggest that the salts present in the extract stabilize the enzyme.
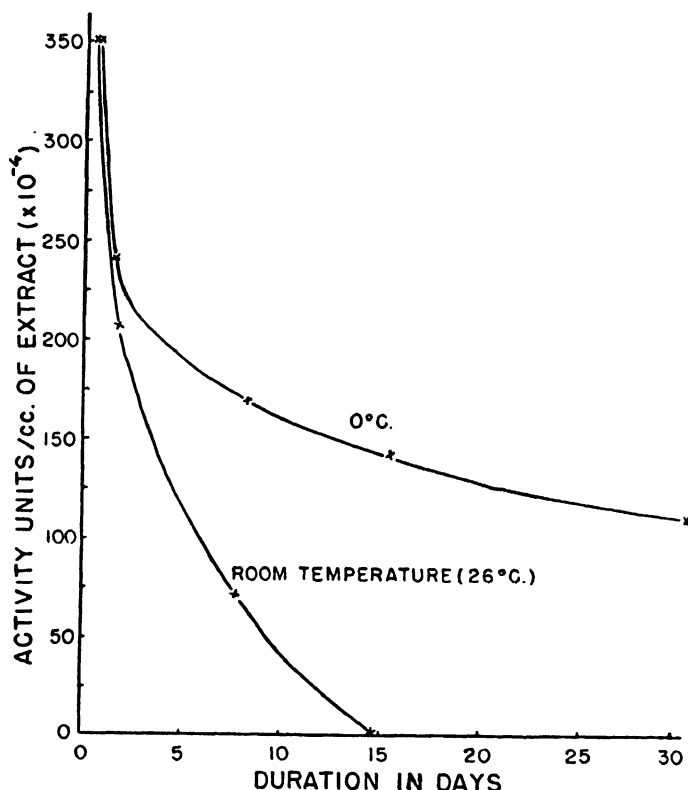
FIG. 5. Stability of dialyzed enzyme with time.

Effect of Cations on Enzyme Activity

The activities of alfalfa and orange pectinesterases have been shown to be influenced markedly by mono- and divalent cations (8, 4). Activation of tomato pectinesterase by NaCl has also been reported recently (9, 10). Since increased activity caused by cations depends primarily on the pH of the reaction mixture, their effects on tomato pectinesterase was studied at 4 different pH values. Activity measurements were made as usual in appropriate buffer solutions containing

varying amounts of calcium and sodium ions and used as their chlorides. A 24-hour dialyzed preparation of the enzyme was employed for these studies.

The results (Figs. 6 and 7) show that the effect of cations on enzyme action is more pronounced at lower pH values, while at or near the optimum pH for the enzyme there is very little increase in activity
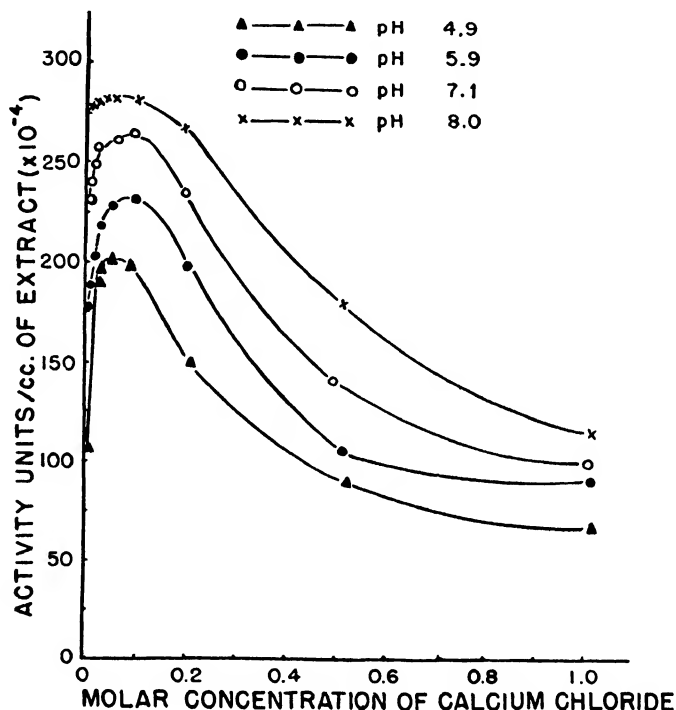
FIG. 6. Effect of divalent cations.

brought about by cations. Further, the activities at lower pH values for any particular salt concentration are always less than the activity at optimum pH without added salt.

The foregoing results are in general qualitative agreement with the earlier observations (4, 8, 9, 10). However, the activity of tomato pectinesterase at lower pH value in presence of optimum concentration of cations was only twice as much as in their absence. The enzyme was most active in presence of 0.01–0.1 M CaCl$_2$ at all pH values, while

higher concentrations caused inhibition. At pH 4.9, the concentration of NaCl required for maximum activity was greater than that of divalent cations, *viz.* 0.5 M, but at higher pH values this concentration caused inhibition. Thus, the concentration of salt required for maximum activity fell to a minimum as the pH approached the optimum. With divalent cations, this change was not so pronounced. The latter
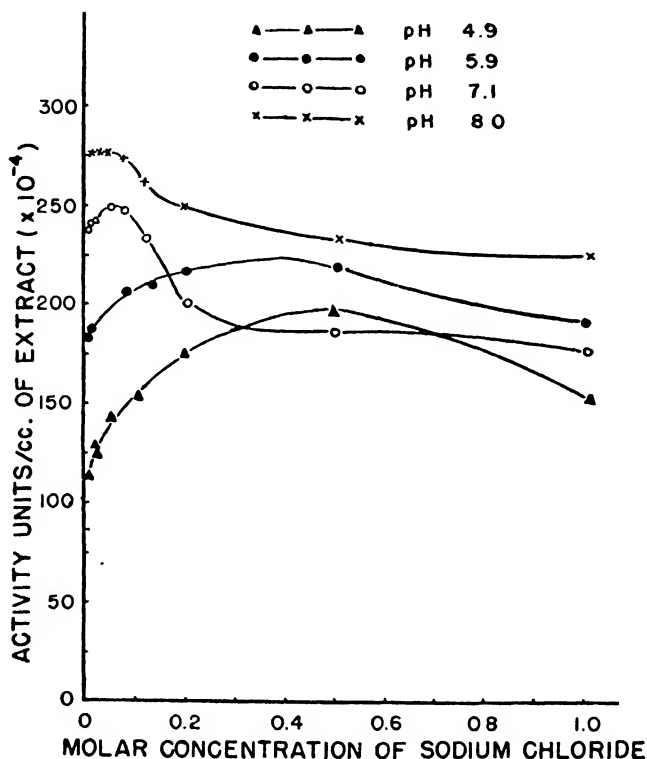
FIG. 7. Effect of monovalent cations.

gave an optimum plateau at all pH values, whereas this was so with monovalent cations only at lower pH values.

Clarification of Fruit Juices with Tomato Pectinesterase

The turbidity in fruit juices is due to their content of pectin and other colloids (20). Natural clarification of fruit juices takes a considerable amount of time, but this can be accelerated by the addition

of pectic enzymes which bring about hydrolytic degradation of pectin. In Table III are given the results on the clarification of orange juice with tomato pectinesterase extract. The fruit juice, obtained by expression in a hand reamer, was filtered through muslin and used, either as such or after dilution, for clarification studies with added pectinesterase solutions. Turbidity was measured with the Klett-Summerson photoelectric colorimeter, and the results are expressed as per cent transmittance of light.

TABLE III

Enzymic Clarification of Orange Juice

Details	pH	cc. of enzyme extract used/100 cc.	Per cent transmittance at the end of		
			1 hr.	4 hrs.	24 hrs.
Original juice	3.75	1.0	0	56.6	98.5
Original juice	3.75	2.5	0	57.2	98.4
(× 2) diluted juice	4.0	1.0	0	36.8	66.6
(× 2) diluted juice	4.0	2.5	0.74	42.1	77.6
(× 4) diluted juice	4.2	1.0	0	6.2	55.7
(× 4) diluted juice	4.2	2.5	1.63	20.0	65.6

It may be seen that clarification is more rapid with the undiluted fruit juice than after dilution, in spite of the fact that addition of enzyme relative to the pectin present in the fruit juice is more in the latter case. These results may be explained as due to the concentration of the salts in the fruit juice, which is an important factor in determining the activity of the pectinesterase at the low natural pH of the fruit juice.

Since tomato pectinesterase is invariably associated with polygalacturonase (5), the latter may also be expected to promote clarification of the fruit juice in the above experiments, particularly because of the pH being nearer its optimum. As, however, the degree of clarification does not increase with dilution, it may be inferred that the quantity of polygalacturonase in the pectinesterase preparation is only small and that clarification is brought about mainly by the pectinesterase. The relative hydrolytic action of pectinesterase and polygalacturonase when present together and at various pH values will form the subject of a subsequent communication.

DISCUSSION

The studies reported here emphasize the importance of the complex relationship between pectinesterase activity, pH and salt concentration shown by Lineweaver *et al.* (8). The effect of cations on tomato pectinesterase (*cf.* Figs. 6 and 7) cannot be taken as representing their true relationship to deesterification. The citrus pectin used as substrate in this study had an ash content of 1.15%. Further, for activity measurements at higher pH values, the pectin solution had always to be neutralized, thus contributing additional small salt formation while the various buffers used at different pH values also furnished some cations. Hence, the buffer-substrate mixture invariably contained small varying amounts of cations prior to addition of either NaCl or $CaCl_2$. This may, to a small extent, be responsible for the low increased activity of tomato pectinesterase in presence of di- or monovalent cations at different pH values as compared to the observations of Lineweaver *et al.* (8) and MacDonnell *et al.* (4) for alfalfa and orange pectinesterases, respectively, and would also account for some variations from the values more recently reported for the effect of NaCl on tomato pectinesterase activity (9, 10). Besides this difference in regard to the effects of cations, tomato pectinesterase varies from alfalfa and citrus pectinesterases (8, 4) in its stability, particularly at higher temperatures, optimum temperature, and pH, indicating the wide differences that may be possible in the nature of the enyzme from different sources (4, 9, 10).

SUMMARY

(1) Pectinesterase activity in tomato was determined by estimating the amount of methanol liberated from pectin under standard conditions.

(2) The enzyme is best extracted from the pulp with 0.1 M Na_2HPO_4 at pH 8.

(3) An active enzyme concentrate or a dry preparation can be obtained by precipitating the enzyme with 50% ammonium sulfate from a buffer extract above pH 7, preceded by discarding the initial precipitate with 20% concentration of salt.

(4) The optimum conditions for enzyme activity were found to be pH 7.8 and 50°C. for 10 minutes' reaction time. The enzyme is un-

stable below pH 6 and at ordinary temperature and is completely and immediately inactivated at 80°C.

(5) The effects of salts on the stability of the enzyme and of mono- and divalent cations on the activity at different pH values are well marked.

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Preliminary Observations on Using a Synthetic Milk for Raising Pigs from Birth [1,2,3]

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INTRODUCTION

Purified rations were first employed successfully in experimental feeding of swine by Chick *et al.* (1). Wintrobe *et al.* (2) demonstrated the need for thiamine by use of synthetic diets. A year later Wintrobe (3), using younger pigs, reported very low gains by animals fed a synthetic diet during the first 2–3 months of life. Cunha *et al.* (4), using synthetic rations, produced weight gains approaching those made by pigs self-fed on a natural grain ration containing 30% fish meal. Briefly, the literature on the nutritional requirements of swine has shown that these animals also require vitamin A (5), vitamin D (6), nicotinic acid (7), riboflavin (8), pantothenic acid (8), and pyridoxine (10). McRoberts and Hogan (11) found no evidence that the pig required vitamin E, vitamin K, choline, biotin, inositol and *p*-aminobenzoic acid. On the other hand, Cunha *et al.* (12) found that pigs farrowed by and weaned from sows on a choline-deficient ration and then placed on a choline-deficient diet grew very poorly and developed a very rough hair coat, and fatty livers. Cunha *et al.* (13) showed that the pig needs biotin when desiccated egg white is fed in the ration, and Lindley and Cunha (14) showed a need for biotin and inositol by the pig when sulfathalidine was included in the ration. Cunha and collaborators (4) failed to show any beneficial effect on growth, efficiency of food utilization, or external appearance of pigs fed a purified diet supplemented with folic acid or *p*-aminobenzoic acid. However, these workers found that folic acid (pteroylglutamic acid) and *p*-aminobenzoic acid were of some help in stimulating hemoglobin formation.

Clark (15) developed a method for making synthetic milk. Wintrobe (3) started using a synthetic milk but abandoned it after finding that pigs a few days old learned to take gruel from a shallow crock or trough. A synthetic milk was used for pigs by McRoberts and Hogan (11). In the work described by them, newborn pigs were removed from the sows at birth and were placed on a basal diet composed of casein, sucrose, corn starch, lard, and minerals, supplemented with the 4 fat-soluble vitamins and 6 members of the B-complex group. Failure of the pigs to receive colostrum was concluded to be at least partly responsible for their early death. These workers then allowed the pigs to suckle their dams for the first 2 days after birth. They found that few pigs survived and attributed the cause to a lack of some unrecognized factor. Gamble, Earle and Howe (16) reported the successful use of blood serum as a colostrum substitute for foals when fed reconstituted, enriched, dried cows' milk. Wiese and others (17) reported the development of a liquid synthetic diet which was satisfactory for the nutrition of the young dairy calf when supplemented with all the known vitamins, and with high levels of vitamin A and anti-scour serum given immediately on receiving them.

The data reported herein are further observations on an attempt to raise pigs from birth on a synthetic milk. In this work was used a purified ration containing all known vitamins, various sulfonamides, crude casein and a liver preparation, lactose, penicillin, Kaopectate, with blood serum and plasma as colostrum substitutes.

Experimental

Baby pigs farrowed by 6 crossbred sows (Chester White × Danish Landrace) which had been bred to a Danish Landrace boar were used as experimental animals. The dams of these pigs were fed an adequate College Herd sow ration. At birth the litters were divided into groups of 2 or 3 pigs each and were placed in screened cages (Fig. 1) approximately 2 × 3 feet in size and similar in construction to those described by Green et al. (18). These cages were located in a new, disinfected room maintained at a temperature of 22°C.

The pigs were taught to drink right after birth from infant nursing bottles equipped with rubber nipples. Within the first 6 hours of life all pigs received orally 55,000 I.U. of vitamin A, 5,500 I.U. of vitamin D, and 300 mg. of ascorbic acid. The synthetic milk used was compounded to simulate sow's milk as reported by Hughes and Hart (19) and modified to contain 15.71% solids. The composition of the synthetic milk used is given in Table I.

The synthetic milk used in these experiments was prepared by a method similar to that used by Wiese et al. (17). However, smaller quantities were prepared daily in the laboratory. In the preparation of the casein solution, enough sodium bicarbonate was added to obtain a pH of 6.8–7.0 in the final mixture. The mixing operation was performed with a Waring Blendor (at approximately 4000 r.p.m.). Although considerable foaming of the mixture occurred, the foam disappeared on cooling or on warming while stirring. The minerals were added in a manner similar to that used by Wiese et al. (17). Homogenization of the lard into the milk was found to be as complete using

TABLE I

Composition of Synthetic Milk Used

	1	2
Vitamin test casein[a]	39.6	
Crude casein		39.6
Sucrose	25.5	
Lactose		25.5
Salt mixture[b]	6.2	6.2
Lard	28.7	28.7

	Salt mixture g.	Vitamins fed	Level/kg. body wt. daily
NaCl	335.0	Thiamine	1.04 mg.
$K_2HPO_4 \cdot 3H_2O$	845.0	Riboflavin	0.24 mg.
$Ca_2H_2(PO_4)_2 \cdot 4H_2O$	190.0	Niacin	2.40 mg.
$MgSO_4 \cdot 7H_2O$	204.0	Pyridoxine	0.40 mg.
$CaCO_3$	600.0	Pantothenic acid	1.00 mg.
$Fe_4(P_2O_7)_3 \cdot 9H_2O$	35.0	Choline	20.00 mg.
KI	1.6	Folic acid	0.034 mg.
$MnSO_4 \cdot 4H_2O$	2.8	Biotin	0.013 mg.
$ZnCl_2$	0.6	Inositol	67.00 mg.
$CuSO_4 \cdot 5H_2O$	0.6	PABA	6.70 mg.
$CoCl_2$	0.4	Ascorbic acid	75.00 mg.
		Vitamin K	2.00 mg.
		Vitamin E	10.00 mg.
		Vitamin A	2500 I.U. daily
		Vitamin D	250 I.U. daily

[a] Manufactured by General Biochemicals, Inc., Chagrin Falls, Ohio.

[b] Salt mixture of Phillips and Hart (23) with added manganese and cobalt.

the Waring Blendor (15 minutes at 60°C.) as was attained using the homogenizing equipment available. The use of the Waring Blendor for the process is practical only in the preparation of relatively small amounts of the synthetic milk. After homogenization, the milk was pasteurized at 60°C. for 30 minutes, cooled, and transferred to sterile containers and stored in the refrigerator at 3°C.

During the first week the pigs were fed at 2-hour intervals with a gradual decrease in the number of feedings until the third week, when a 3-hour interval was used. McRoberts and Hogan (11), in their work with a synthetic milk, fed their pigs every 3 hours. Hughes and Hart (19), in a study on sow's milk, allowed the small pigs to suckle every 2 hours in the early stages of lactation. The synthetic milk used at the barn was kept in an ice box and was heated to 37°C. before feeding.

Diets given, vitamins fed, and therapeutics administered to the various lots are described in Tables I and II. Homologous serum and plasma were used as colostrum substitutes. The serum and plasma were obtained from the blood of healthy animals raised under similar environmental conditions as the dams of the pigs used in these experiments. The blood used for serum was collected in sterile containers and allowed to coagulate overnight after which the serum was removed aseptically with a pipette

TABLE II

Treatments Used, Length of Survival, and Gross Pathology Obtained

Lot	No. of pigs	Colostrum substitute	Ration type	Therapeutic measures	Pig no.	Length of life (days)	Length of life (av.)	Post mortem
I	3	No serum or plasma	Basal*		1 2 3	2+ 1+ 1+	1+	1. Gastritis and duodenitis 2. No recognizable lesions 3. Peritonitis
II	3	20 ml. serum *per os*	Basal		4 5 6	6+ 9+ 4	6	4. Pneumonia 5. Gastritis and typhlitis 6. No recognizable lesions
III	3	20 ml. serum intraperit.	Basal	Sulfathalidine given to all pigs starting on fourth day.	7 8 9	17 12+ 7+	13	7. No recognizable lesions 8. Enteritis in anterior small intestine 9. Pneumonia
IV	3	10 ml. serum intraperit. 10 ml. serum *per os*	Basal	Anti-pernicious anemia liver preparation given to pigs No. 11 and 12 starting on third day.	10 11 12	3+ 8+ 4	7	10. Pleuritis and typhlitis 11. Pneumonia and enteritis 12. Gastro-enteritis
V	3	20 ml. plasma *per os*	Basal	Penicillin given to pig No. 15 starting on tenth day.	13 14 15	21+ 4+ 22+	17	13. Slight hemorrhagic colitis and typhlitis 14. Severe lung congestion, pleural adhesions, and pericarditis 15. Hemorrhagic colitis, typhlitis and gastritis
VI	3	10 ml. plasma *per os* 10 ml. plasma intraperit.	Basal	Penicillin given to pig No. 17 starting on tenth day.	16 17 18	11 11 21	15	16. Pneumonia 17. Slight enteritis 18. Hemorrhagic proctitis and typhlitis
VII	2	10 ml. plasma intraperit. 50 ml. plasma *per os*	Lactose + crude casein	Sulfathalidine given to both pigs right after birth in milk.	19 20	4 5+	5	19. Enteritis 20. Slight pleuritis and kidney petechia

TABLE II—*Continued*

Lot	No. of pigs	Colostrum substitute	Ration type	Therapeutic measures	Pig no.	Length of life — days	Length of life — av.	Post mortem
VIII	2	as above	Lactose + crude casein	Sulfathalidine started on the sixth day replaced by sulfamethazine on the eleventh day. Kaopectate started on the fourteenth day to both pigs.	21 22	15 16	15+	21. Nephritis, colitis, and lung hemorrhage 22. Nephritis, pleuritis, and pericarditis
IX	2	as above	Lactose, crude casein + 1-20 liver powder	Kaopectate given to pig No. 23 on the sixth day.	23 24	8 4	6	23. Lung congestion with pleuritis 24. Kidney petechia and hemorrhagic pneumonia
X	2	10 ml. plasma intraperit. 50 ml. plasma *per os*	Lactose + crude casein		25 26	4 3	3+	25. Urates in kidneys, ureters, and bladder 26. Gastritis, pericarditis, and kidney petechia
XI	2	plasma levels as above but from inoculated pig	Lactose, crude casein 1-20 liver powder + brewers' yeast		27 28	4 6	5	27. Urates in kidneys, ureters, and bladder 28. Urates in kidneys, ureters, and bladder
XII	3	Kept 3.5 days with mother	Basal		29 30 31	19 21+ 17	20	29, 30, 31. Orchitis, nephritis with petechia and medullary hyperemia, hemorrhage lymphadenitis, light yellow liver, hemorrhagic colitis and typhlitis

ᵃ Basal is the ration shown in Table I containing purified casein, sucrose, lard, minerals, and all known vitamins.

and place in sterile flasks and kept in a refrigerator. The blood for plasma was collected in 250 cc. flasks, using 2 mg. of potassium oxalate/ml. of blood, as recommended by Coffin (20). The plasma was separated from the oxalated blood by centrifuging for 5 minutes at 1700 r.p.m. and stored in a manner similar to the serum.

One healthy 6-month old pig was injected intradermally with a bacterin prepared from the organisms contained in the feces excreted by 2 pigs fed the synthetic milk, and which had developed a severe diarrhea. Plasma was collected from this animal in the method described above and used with the pigs in Lot XI as a colostrum substitute. This was tried in an effort to determine whether the plasma from this pig would be more efficacious in preventing the diarrhea which always developed with pigs fed the synthetic milk.

In the first phase of the experiment, a comparison between serum and plasma as replacements for the antibodies found in colostrum was made, using various methods of administration. Length of survival of the pigs in the first phase of experimental work indicated that plasma was superior, and, as a result, plasma was used exclusively in the remainder of the experiment. The method of administration of plasma in the latter phase was as follows: 10 ml. of plasma was injected intraperitoneally immediately after birth. During the first 24 hours each pig was fed an additional 30 ml. of plasma incorporated in the milk, followed by 10 ml. on the second and third days, also fed in the milk.

In an effort to alleviate the severe diarrhea exhibited by all pigs, various therapeutic measures were tried. These included adding sulfathalidine to the milk at levels of 0.5 and 4.0% of solids, sulfamethazine at levels of 1 grain/lb. body weight/day fed in the milk, penicillin in oil at the rate of 5000 units/day given intramuscularly, and Kaopectate (Upjohn) at levels of 2 g./pig/day fed in the milk.

When lactose and crude casein were used they were substituted in equal amounts for sucrose and purified casein respectively. 1–20 Liver powder (Wilson's) was included in the synthetic milk at a level of 2% of the dry matter. Brewers' yeast powder (Mead Johnson & Co.) was incorporated in the milk at a level of 2 g./pig daily. The anti-pernicious anemia liver extract (Lederle Laboratories—type porcine) was injected intramuscularly in the gluteal region at a level of 0.2 cc. (2 U.S.P. units)/pig daily.

Post mortem examination was accomplished on all pigs to determine pathological lesions.

RESULTS AND DISCUSSION

The results obtained on length of survival and gross pathology are shown in Table II. The methods used and rations fed were inadequate to maintain life and produce weight gains (Table III) for an extended period of time.

The data shown in Table II indicate that pigs fed no colostrum substitute (lot I), such as serum or plasma, died very shortly after birth. This finding indicates that colostrum and serum or plasma contain something which is necessary for the survival of the pig. The anti-

bodies which serum and plasma are known to contain are very important but there may be another substance or substances present which is also necessary for maximum survival. Gamble, Earle and Howe (16), using serum as a substitute for the antibodies, were successful in raising foals which have the same number of membranes separating the fetal and maternal blood as the pig. McRoberts and Hogan (11) were able to raise a few baby pigs using a synthetic milk supplemented by crude carriers, but only after the pigs had received colostrum for 2 days.

In the first phase of this experiment, where the basal synthetic milk was used, it was found that the pigs given plasma survived for a longer period of time than the pigs given serum. Following these first trials, plasma was used entirely and higher levels were administered.
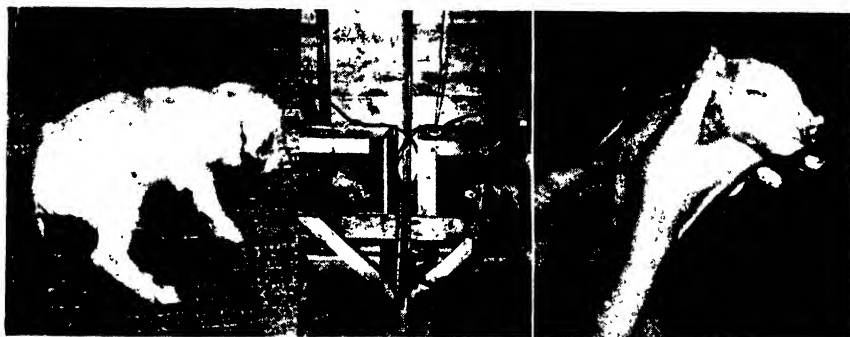
FIG. 1. Left, shows a pig with severe diarrhea. Center, is the type of pens in which the pigs were kept. Right, is a pig with a dark exudate around the eyes and with the eyelids cemented shut.

In the preliminary trials (Lots I–VI) it was found that a severe diarrhea (Fig. 1) developed with all pigs within 12–36 hours after birth. None of them recovered from this disorder. The pigs gradually became unthrifty in condition and developed a dark exudate around the eyes, which often cemented the lids together (Fig. 1). The above findings are similar to the results obtained by McRoberts and Hogan (11).

The preliminary results obtained indicated that the basal synthetic milk was inadequate, and, since McRoberts and Hogan (11) suggested that some unrecognized factor was needed for the very young pig, crude casein, 1–20 liver powder, and brewers' yeast were tried as

part of the ration in subsequent experiments. In addition, lactose was substituted for sucrose, since it is the sugar found in milk. The results obtained with the pigs in Lots VII–XI showed that the addition to the ration of crude casein, lactose, 1–20 liver powder or brewers' yeast was of no apparent benefit in prolonging life. However, the test for brewers' yeast was not adequate since the yeast was not fed until the 2 pigs were 2 days old and they died at 4 and 6 days of age, respectively.

The pigs in Lot XI were given plasma from the pig which had been inoculated with a bacterin made from organisms in diarrheic feces. The plasma gave no relief in the scours developed by the pigs. If anything, they scoured sooner than the other pigs on experiment.

In an effort to combat the diarrhea which invariably developed, the following therapeutic agents were used: penicillin, sulfathalidine,

TABLE III
Average Weight Gains of Pigs

Lot	Birth weight g.	Weight gain or loss in g. for 23 days									
		3rd	5th	7th	9th	11th	13th	15th	18th	21st	23rd
I	1023	−21[a]									
	1053										
	1189	−82									
II	787	100[b]	157								
	900	50	87	62	−299						
	562	40									
III	956	133	106	141	65	52	286	−43			
	900	54	157	184	−28	−232					
	1125	−8	95	−120							
IV	1089	−239									
	1220	−166	90	−29	·						
	1158	−224									
V	1237	64	207	87	100	−14	44	−14	−14	−31	
	956	38	−172								
	1125	41	9	123	−38	2	1	102	18	−38	−167
VI	1100	−99	48	168	−37	−190					
	956	67	63	46	78	−75					
	1125	88	90	127	102	13	−58		51	−136	

TABLE III—*Continued*

Lot	Birth weight $g.$	Weight gain or loss in g. for 23 days									
		3rd	5th	7th	9th	11th	13th	15th	18th	21st	23rd
VII	778	−38	−58								
	936	−46	−15								
VIII	1594	154	71			459	−218	−250			
	1584	−104	206			500	11	−287			
IX	926	24	76	−56							
	830	−30	−44								
X	1510	252	−72								
	1143	−156									
XI	1608	64	−10								
	1478	−60									
XII	675	439	303	114	58	219	127	37	143		
	1012	205	449	−45	177	252	158	4	174	−121	
	956	272	262	−79	97	117	150	−70	−112		
Pigs kept with sows during entire period	675	223	258	377	228	460	440	560	480	720	360
	956	412	220	429	337	540	540	360	630	900	450
	1125	453	435	436	364	554	460	460	790	540	630

[a] A negative sign indicates a loss in weight.
[b] No sign indicates a gain in weight.

sulfamethazine, Kaopectate, and an anti-pernicious anemia liver preparation. These therapeutic measures failed to control the diarrhea or noticeably change the appearance of the pigs. This suggests that the cause of death was most likely due to improper nutrition or, at any rate, not caused by microorganisms within the range of penicillin or the sulfonamides. Kaopectate administration was observed to initially increase the consistency of the watery feces but failed to relieve the diarrheic condition.

Three pigs were left with the dam for 3.5 days and then placed on the basal synthetic milk. They grew well for a period of 12–15 days but succumbed at an average of 20 days after birth. These pigs did not develop the diarrheic condition until about 6 days after having been

TABLE IV

Average Feed Consumption/Pig Daily in Ml. of Synthetic Milk

Lot	Days										
	1	2	3	4	5	6	7	10	14	17	21
I	15	205	120	5							
II	225	223	216	251	185	210	205				
III	198	280	310	370	315	410	305	270	175	25	
IV	110	190	245	190	185	160	200				
V	130	260	210	360	290	380	325	285	280	440	435
VI	125	235	200	285	360	365	280	240	205	440	
VII	90	205	185	165	95						
VIII	320	275	295	390	340	445	390	490	150		
IX	135	265	255	355	380	330	280				
X	350	410	395								
XI	360	400	340								
XII	With sow 3.5 days, then fed synthetic milk				10	245	370	530	360	460	435

transferred from the dam to the synthetic milk. In other words, having suckled the dam for 3.5 days resulted in the pigs having more resistance to the development of the diarrheic condition.

Post-mortem findings are shown in Table II. Gastroenteritis and pneumonia were the most common lesions observed at necropsy.

The pigs left with the dam for 3.5 days before being fed the synthetic milk developed the most severe symptoms obtained in this experiment. A bloody diarrhea, hemorrhagic colitis and typhlitis, kidney petechia, yellow livers, hyperemic testicles and hemorrhagic lymph nodes were observed with the 3 pigs.

It is interesting to note that of the 31 pigs fed on synthetic milk 3 showed urates in the kidneys, ureters, and in the bladder. All 3 pigs were farrowed by the same sow. On the other hand, of the pigs that were left with the experimental sows, 5 of the 6 pigs that died showed urates in the kidneys, ureters, and bladder. Cunha *et al.* (21) found urates in the kidneys of small pigs farrowed by sows on a corn-soybean oil meal ration known to be deficient in some unknown factor or factors. Typical cases of uremia have a considerable amount of yellow to orange-colored precipitate in the kidneys, ureters and bladder according to

Madsen *et al.* (22). These workers found that withholding food entirely, or limited feeding of baby pigs, resulted in the production of a condition that was similar to acute uremia as encountered under herd conditions.

Two pigs were kept on the synthetic milk and plasma from birth until 3.5 days of age. They were then returned to the mother. Both pigs suckled but died within 2 days. Autopsy revealed urate deposits in the kidneys and bladder. Since urates were obtained with these pigs, it is difficult to evaluate whether or not it might be possible for pigs to survive on the mother after having been kept away for their first 3.5 days of life.

The average weight gains and milk consumption are given in Tables III and IV. It is felt that these data will be of value to investigators who contemplate experimental work using a synthetic milk for pigs.

ACKNOWLEDGMENTS

SUMMARY AND CONCLUSIONS

Pigs removed from the mother at birth and placed on a synthetic milk containing all known vitamins and with plasma or serum as colostrum substitutes failed to survive longer than 22 days.

It was found that pigs fed no colostrum substitute such as serum or plasma died very shortly after birth. This finding indicates that colostrum and serum or plasma contain something which is necessary for the survival of the pig. Indications are that plasma was superior to serum as a colostrum substitute for the new-born pig.

A severe diarrhea developed with all pigs fed synthetic milk. The pigs gradually became unthrifty in condition and developed a dark exudate around the eyes which often cemented the lids together.

The results obtained indicate that the ration used was inadequate. The addition to the ration of crude casein, lactose, 1–20 liver powder,

or an anti-pernicious anemia liver extract was of no apparent benefit in prolonging life or improving the appearance of the pigs.

The use of the following therapeutic agents was of no benefit in controlling the diarrhea obtained, or in prolonging life: penicillin, sulfathalidine, sulfamethazine, and Kaopectate.

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Cytochrome c in Epidermal Carcinogenesis in Mice Induced by Methylcholanthrene [1]

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INTRODUCTION

This paper presents the results of an investigation of the cytochrome c content of the epidermis of mice undergoing carcinogenesis induced by methylcholanthrene. A transplantable squamous cell carcinoma originally derived from the epidermis by the application of this carcinogen was also studied (1). Reports of the cytochrome oxidase, succinic dehydrogenase (2), and adenylpyrophosphatase (3) activities in similar tissues have been made previously.

METHOD

A polarographic procedure capable of detecting minute quantities of cytochrome c (4) was developed, as only small amounts of tissue are available for analysis (2). Although the polarographic method was easily adapted to the determination of cytochrome c in mouse heart, kidney, liver, and skeletal muscle (4), some difficulty was experienced in applying the procedure to mouse epidermis. In this tissue the cytochrome c is firmly attached to another substance which interfered with the catalytic wave. For this reason, the cytochrome c content of the epidermis was also determined spectrophotometrically with a Beckman spectrophotometer. The cytochrome c was purified for polarographic analysis by adsorption on aluminum oxide (4). The cytochrome c from epidermal extracts was adsorbed twice, and the eluate from the second adsorption was brought to pH 6.8–7.0, centrifuged, and the supernate containing the cytochrome c was made up to 10 ml., and an aliquot used for polarography. For spectrophotometry the eluate after the first adsorption on aluminum oxide was adjusted to pH 5.8–6.0, centrifuged, and cytochrome c reprecipitated in a 15 ml. centrifuge tube with trichloroacetic acid and $(NH_4)_2SO_4$ (5). The protein containing the cytochrome c was dissolved in a few drops of water, brought to pH 7.2–

7.4 with 0.2 N NaOH, and then made to 1 ml. with 0.1 M sodium phosphate buffer of pH 7.4. After centrifugation the cytochrome c content in the optically clear solution was determined at 5500 Å following reduction with $Na_2S_2O_4$. This adsorption and elution from aluminum oxide permits purification of cytochrome c, and also makes possible the concentration of a small quantity of the latter from large samples of tissue which are low in this enzyme.

Nature of the Catalytic Wave of Cytochrome c from Epidermis

A polarogram of pure cytochrome c (6 γ/ml.) is shown in Fig. 1. The wave was obtained in a solution which contained 1 N NH₄OH, 1 N NH₄Cl, 1×10^{-3} M hexammino cobaltic chloride, and a trace of gelatin as a maximum suppressor. The cytochrome c (0.411% Fe) used for calibration purposes in this study was electro-
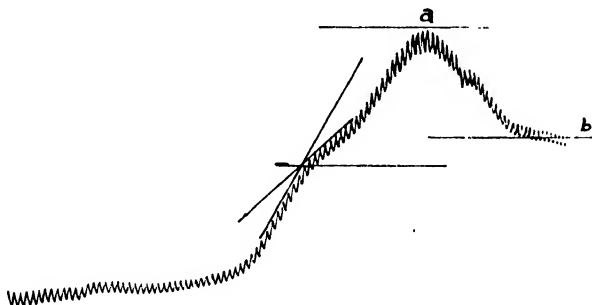
Fɪɢ. 1. Polarogram of pure cytochrome c (6 γ/ml.).

phoretically prepared and kindly given to us by Dr. H. Theorell of the Biochemical Institute of the Medical Nobel Institute, Stockholm, Sweden. The mixture was polarographed immediately, starting at -1.0 volt (vs. the saturated calomel electrode) in an open shell vial which permitted deep insertion of the mercury electrode. The tip of the catalytic wave is about -1.4 volts vs. the saturated calomel electrode. The catalytic wave immediately follows the reduction of the cobaltous ion, and, after rising steeply to a maximum, drops rapidly, terminating only slightly above that of
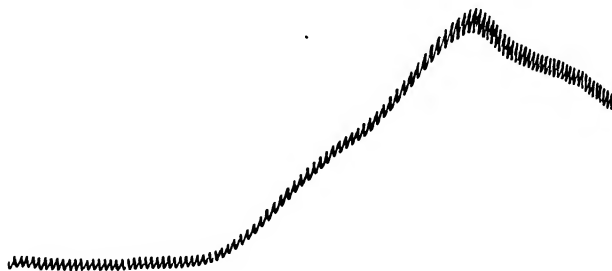
Fɪɢ. 2. Polarogram of cytochrome c extracted from mouse epidermis.

the inflection due to the reduction of the cobaltous ion. Perfect catalytic waves for cytochrome c are obtained from rat and mouse heart, kidney, skeletal muscle and liver (4), but in the case of epidermis the waves are not ideal. A polarogram of cytochrome c from epidermis is shown in Fig. 2. The wave is not perfect since the descending portion of the catalytic wave does not drop nearly to the level of the inflection of the reduction of the cobaltous ion. Some other substance associated with the cytochrome c having similar adsorptive properties on aluminum oxide is reduced at a potential immediately following that of the reduction of the cobalt ion. This reduction raises the catalytic hump considerably, and necessitates another method of calibration.
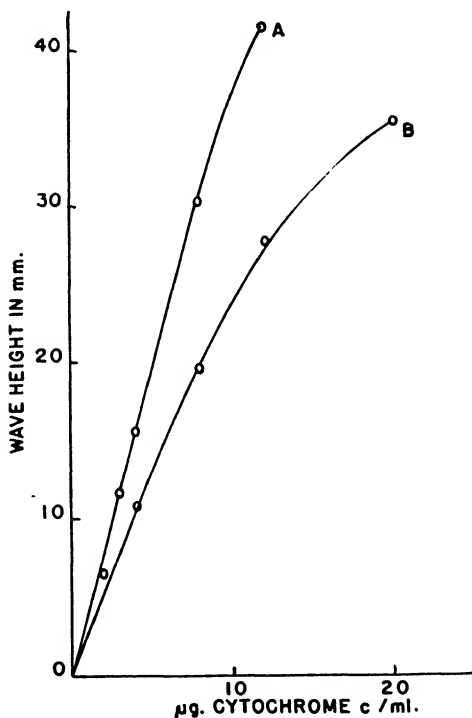
FIG. 3. Calibration curves of the catalytic wave of cytochrome c in N NH$_4$OH, N NH$_4$Cl, and 1×10^{-3} M hexammino cobaltic chloride.

In the regular method of calibration the height of the catalytic wave was measured from the tip of the wave to the point of inflection of the reduction of the cobalt ion (4). This was done by drawing a line contiguous with, and parallel to, that of the rise of the cobalt wave, and a line in a similar fashion through the first portion of the catalytic wave. At the point of intersection of these two lines a line was drawn parallel to the tip of the catalytic wave and the distance in mm. was measured with a caliper (Fig. 1.). This calibration curve is shown in Fig. 3, Curve A. The concentration of the enzyme is proportional to the wave height up to about 30 γ/ml.

For the cytochrome c in epidermal extracts another method of calibration was employed to correct for the reduction of the interfering material which was carried along with the cytochrome c during adsorption on aluminum oxide. This curve was obtained by measuring the height of the catalytic hump from its tip (Fig. 1, a) to a point at -1.7 volts vs. the saturated calomel electrode (Fig. 1,b). The latter point of measurement can be taken at the lower or upper part of the oscillations of the galvanometer on the polarogram. The distance between a and b is plotted against the concentration of pure cytochrome c to give the calibration curve shown in Fig. 3, Curve B. The concentration of the enzyme is proportional to the wave height (to about 12 γ/ml.).

A comparison of the results on the cytochrome c content of epidermis undergoing carcinogenesis with the polarographic and spectrophotometric method is shown in Table I. The values, expressed as γ of cytochrome c/g. fresh tissue, agree fairly well.

TABLE I
Cytochrome c in Carcinogenesis

Treatment	No. of mice	γ Cytochrome c/ g. tissue (wet weight) Spectrophoto-metric	No. of mice	γ Cytochrome c/ g. tissue (wet weight) Polarographic
Control	116	57	77	46
Benzene control	124	58	39	51
Methylcholanthrene				
3 paintings	82	39	20	46
6 paintings	57	68	39	56
12 paintings	60	48		—
18 paintings	60	53	54	42
24 paintings	56	53	71	46
Carcinoma		34		34

Different samples of epidermis were used for each method of determination because of the small quantity of tissue available. There is no appreciable change in the content of cytochrome c in hyperplastic epidermis compared with normal, but there is a decrease of about 30% in the carcinoma by both methods of determining the enzyme (Table II).

DISCUSSION

The content of cytochrome c in practically all neoplastic tissues is less than in the tissue of origin (6, 7, 8). We have likewise found that a transplantable squamous cell carcinoma, originally induced in the epidermis of a Swiss mouse by methylcholanthrene (1), contains less of the compound than does normal epidermis. Moreover hyperplastic

TABLE II

Cytochrome c in Carcinogenesis
Spectrophotometric

Tissue	γ Cytochrome c/ g. of tissue (wet weight)	Per cent change from normal
Control + benzene control	55	—
Hyperplastic	52	—
Carcinoma	34	−38

Polarographic

Control + benzene control	49	—
Hyperplastic	55	—
Carcinoma	34	−31

epidermis contains about the same amount of cytochrome c as does normal and benzene-treated epidermis, although late hyperplastic epidermis was found to contain twice the activity of cytochrome oxidase as normal (2).

The polarographic procedure for the determination of cytochrome c gave values which were in agreement with the spectrophotometric method. A fact of great interest revealed by the polarographic technique is that cytochrome c in the epidermis of mice is closely associated with, or firmly bound to, another substance which cannot be separated from the cytrochrome c by adsorption on aluminum oxide. This is in direct contrast to cytochrome c in mouse heart, kidney, and skeletal muscle, and rat heart and skeletal muscle, in which tissues the cytrochrome c is separated easily by one adsorption on aluminum oxide. In mouse liver, and in rat kidney and liver, 2 adsorptions are necessary to remove all the interfering material from cytochrome c.

A comparison of the activities of cytochrome oxidase, succinic dehydrogenase, and apyrase, and of the content of cytochrome c in epidermal carcinogenesis, is collected in Table III. The activities of the succinic dehydrogenase and cytochrome oxidase are expressed as Q_{O_2} (mm.^{3} O_2 consumed/mg. wet weight/hr.) and of apyrase as Q_p (γ P $\times$ 22.4/31/mg. wet weight/hr.). The ratio cytochrome c-cytochrome oxidase of normal and early hyperplastic epidermis is the same, but decreases signally in late hyperplastic epidermis. In the tumor the ratio is somewhat less than normal, but greater than that of late hyperplastic epidermis. (The average value of cytochrome c obtained by the polarographic and spectrophotometric methods was used.)

TABLE III

Cytochrome c, Cytochrome Oxidase, Succinic Dehydrogenase,
and Apyrase in Epidermal Carcinogenesis

Tissue	a Cytochrome c $\gamma/g.$	b Cytochrome oxidase Q_{O_2}	c Succinic dehydrogenase Q_{O_2}	d Apyrase Q_p	$\frac{a}{b}$	$\frac{a}{c}$	$\frac{a}{d}$
Normal	52.0	9.9	1.4	8.4	5.3	37.0	6.2
M.C.[a] 3,6,12 paintings	52.0	9.9	1.3	7.8	5.3	40.0	6.7
M.C.[a] 18,24 paintings	49.0	15.9	1.2	8.5	3.1	40.9	5.8
Carcinoma	34.0	7.5	2.9	25.2	4.5	11.7	1.4

[a] Methylcholanthrene.

The ratio cytochrome c-succinic dehydrogenase and cytochrome c-apyrase are practically constant in the normal and hyperplastic stages, but there is a very significant diminution in both in the carcinoma. These results indicate a relative deficiency of cytochrome c in relation to the activities of succinic dehydrogenase and apyrase in the tumor, but a greater deficit appears in the cytochrome c-cytochrome oxidase ratio in late hyperplastic epidermis than in the carcinoma.

SUMMARY

The role of cytochrome c in epidermal carcinogenesis in mice was investigated. The enzyme was determined spectrophotometrically and polarographically and both procedures gave results of same order of magnitude. The cytochrome c content of normal, benzene-treated, and methylcholanthrene-treated epidermis is nearly the same, but there is a decrease of about 30% in the transplantable squamous cell carcinoma. The relationship of cytochrome c to the activity of succinic dehydrogenase, apyrase, and cytochrome oxidase in epidermal carcinogenesis is briefly discussed.

The polarographic method revealed that cytochrome c in the epidermis of mice is closely associated with, or bound to, another substance which cannot be separated from the cytrochrome c by adsorption on aluminum oxide. In contrast to epidermis, the cytochrome c of mouse and rat liver, kidney, skeletal muscle, and heart can be removed by one or two adsorptions on aluminum oxide.

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Biochemistry of Wound Healing. II. Water and Protein Content of Healing Tissue of Skin Wounds

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INTRODUCTION

An understanding of the basic composition of repair tissue is important as a background in studying the biochemistry involved in the process of healing of wounds. Knowledge of changes in the basic composition of tissue at various stages of healing is of value in correlating repair tissue growth with growth of other tissues.

The basic composition of healing tissue has not been extensively studied. Healing skin transplants in rabbits have been analyzed for water and nitrogen by Tammann, Blumel and Roese (1), but we have been unable to find similar studies on actively healing wounds of rats. The importance of studying water in repair tissue lies in the fact that, in general, the more active the tissue from a physiological standpoint, the higher the content of water (13). However, change in water content of tissue may merely reflect replacement by another constituent. The importance of protein in the building of new tissue or regeneration of old tissue is recognized.

This paper is concerned with the water and protein content of repair tissue in normally healing, untreated skin wounds on albino rats of different ages and sexes at various stages of healing.

EXPERIMENTAL

Since the rate of healing of wounds in younger animals has been reported to be more rapid than in older animals (2), two age groups were chosen. Also, since a definite sex difference for both water and protein content of skin has been found (3), equal numbers of each sex were included at each stage studied to determine any influence of sex.

Healthy rats maintained on their normal stock diet of mixed commercial dog checkers were divided into 4 groups as follows:

(a) Young males: Age,   108 days; wt., 312 ± 36[2] g.
(b) Young females: Age,   108 days; wt., 206 ± 14 g.
(c) Old males: Age, 460 ± 57 days; wt., 433 ± 27 g.
(d) Old females: Age, 596 ± 45 days; wt., 330 ± 26 g.

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[1] Present Address: Biochemistry Department, Western Reserve University, Cleveland, Ohio.

[2] Average deviation.

Under anesthesia, a wound was placed on each shoulder of a rat in the manner previously described (4). The sections of skin removed from each rat in wounding, were cut into strips 1–2 mm. wide, combined, placed in a small tared glass cup, covered with a tared watch glass, and the fresh weight determined. The samples were then dried to a constant weight over calcium chloride in a vacuum desiccator as recommended by Wynn and Haldi (3). Moisture was calculated from the loss in weight.

At 3, 6, 9, 12, and 18 days after wounding, animals were killed by etherization, the scabs removed and the wounds measured with calipers (4). The repair tissue was carefully dissected out, the two samples from each animal combined, and moisture determined in the same manner as skin moisture.

The vacuum-dried samples of skin and repair tissue from the moisture determination were subjected to nitrogen determination. A modified micro Kjeldahl method with a H_2SO_4–H_3PO_4 digestion mixture containing copper and selenium as catalysts (5) was used. The digested samples were aerated in a manner similar to that of Sobel, Mayer and Gottfried (6) trapping the liberated ammonia in boric acid. Titrations were made with 0.02 N H_2SO_4 using a mixed indicator of methyl red and bromcresol green. Preliminary experiments showed that for skin, as well as other protein-containing substances, digestion must be continued beyond the clearing stage (7). Prolonged digestion resulted in a loss of nitrogen as found by Dalrymple and King (8). For skin 0, 1, 1.5, 2, and 3 hours digestion beyond the clearing stage gave 6.57, 7.30, 7.45, 7.70, and 6.50% nitrogen, respectively.[3]

A standard time of 1.5 hours heating beyond the clearing stage was chosen for 10 mg. of dried skin and 4 ml. of digestion acid. Fourteen control analyses of a sample of casein (by the above technique) gave consistent results (Std. Dev. ± 2.3%). Protein was calculated by multiplying the nitrogen content by the usual factor of 6.25. Repair tissue was treated in the same manner as skin.

The amount of healing at various stages was calculated by means of the following formula from the areas obtained from measurements of 3 diameters of the wound as described in our previous paper (4). In each animal both wounds were measured separately and the average value used.

$$\frac{A_o - A_t}{A_o} \times 100 = \% \text{ healed}$$

where A_o is equivalent to the area at wounding and A_t is equivalent to the area at the time of analysis. Our experiments were not carried beyond the 18 day stage because healing, as judged by external appearance, was complete at that time.

RESULTS

Data for water and protein content of both skin and repair tissue are presented in Table I with the values expressed in percentages, based on fresh weight. These values are derived from all animals studied, treating them solely on an age and sex basis regardless of the

[3] About 1.5 hours heating were required for clearing.

TABLE I

Water and Protein Content of Skin and of Repair Tissue
Calculated on Basis of Fresh Weight

Age	Sex	Skin				Repair tissue			
		No. of rats	Water	No. of rats	Protein	No. of rats	Water	No. of rats	Protein
			Per cent		*Per cent*		*Per cent*		*Per cent*
Young	All	30	57.8±2.6[a]	29	23.5±1.7[a]	30	80.3±3.3[a]	28	15.1±2.9[a]
Old	All	20	56.1±4.5	17	24.8±2.1	19	80.6±2.3	19	15.1±1.7
Young	Males	15	60.6±2.8	15	24.6±2.2	15	80.1±3.5	15	15.8±2.7
Young	Females	15	55.0±2.3	15	22.6±1.2	15	80.5±3.1	13	14.2±1.9
Old	Males	10	59.3±3.3	9	26.8±2.2	10	80.9±1.8	10	15.7±2.0
Old	Females	10	53.1±6.5	8	22.6±2.1	9	80.2±3.3	9	14.5±1.4
All	Males	25	60.1±3.1	23	25.5±2.2	25	80.4±2.8	25	15.7±2.4
All	Females	25	54.2±3.6	23	22.6±1.5	24	80.4±3.2	22	14.3±1.7

[a] Average deviation.

stage of healing. The water and protein content of skin are practically the same for both young and old rats. The skin of males has a higher water and protein content than that of females. When the data for the influence of sex on the water and protein content of skin are subjected to statistical analysis (12) P is found to be less than 0.01 in either case indicating that the differences are significant. The water content of repair tissue is influenced by neither the sex nor age of the animal (*i.e.*, within the limits here studied) in this overall calculation. Protein, on the other hand, although showing no age difference, tends to be higher in repair tissue of males than females. Since $P = 0.08$, the significance of this difference is doubtful (12).

In Table II are to be found the data (together with their average deviations) for the water and protein content of repair tissue of all animals at various stages of healing. Water content is high and constant up through the twelfth day, but decreases by the eighteenth day, although it is still higher than that of normal skin. Protein in repair tissue shows a progressive increase with healing from the third through the ninth day. On our experiment there was a slight drop at the twelfth day which cannot, however, be considered statistically significant. By the eighteenth day, the protein content of repair tissue shows a further increase although the protein content of normal skin has not yet been attained. Undoubtedly, healing was still progressing as measured by

TABLE II

Water and Protein Content of Repair Tissue of Skin Wounds
(fresh weight basis)

Stage of healing	No. of rats	Water	No. of rats	Protein	Extent of healing
Days		*Per cent*		*Per cent*	*Per cent*
3	9	82.9±0.9	9	12.5±0.8	16±8
6	10	81.4±2.0	10	13.7±0.7	50±5
9	10	81.1±1.2	10	15.2±0.6	84±3
12	10	82.1±1.9	9	14.6±1.6	96±2
18	10	74.7±1.7	9	19.7±1.3	100±0

tissue composition changes, even though the wound was completely healed as judged by external appearances.

Our data on the influence of age or sex on per cent of healing as judged by external measurements of wound area appear in Table III. The data in this table have only been subjected to the simplest of statistical treatment (determination of average deviation), as the numbers involved and the type of material do not warrant more than that. Our data indicate that there is a tendency for the younger animals to exhibit faster healing during the early stages (*i.e.*, 3 and 6

TABLE III

Influence of Age and Sex on Healing at Different Stages

Stage of healing	Age group	No. of rats	Extent of healing	Sex	No. of rats	Extent of healing
Days			*Per cent*			*Per cent*
3	Young	6	21±11	Male	5	11±11
	Old	3	8±4	Female	4	23±12
6	Young	6	55±6	Male	5	48±8
	Old	4	43±3	Female	5	52±7
9	Young	6	84±2	Male	5	84±3
	Old	4	84±5	Female	5	83±4
12	Young	6	98±1	Male	5	97±2
	Old	4	93±1	Female	5	95±4
18	Young	6	100±0	Male	5	100±0
	Old	4	100±0	Female	5	100±0

days), but that this tendency is not apparent at the later stages. The data also indicate that females heal more quickly in the early stages of healing but the difference cannot be considered significant with the small number of animals studied. No sex difference in healing rate is indicated at later stages.

DISCUSSION

Our values for the water content of the skin compare favorably with those of Wynn and Haldi (3) and Haldi, Giddings and Wynn (9). Since the method for determining water was the same in both cases, slight variations encountered are probably due to age and diet. Our protein values are about 10–13% lower for skin than theirs. This may be due to the greater amount of fat in our animals which were older than the ones they used.

The water content of repair tissue in the rapidly healing stages is very high. Compared with the values of Lowrey (10) for other tissues of animals of the same age, repair tissue up through the twelfth day of healing has a water content similar to that of lungs and eyeballs, and higher than that of all other organs except testes. The water content of repair tissue is comparable to such active tissues as embryo and tumor (11). The relative constancy of water from the third through the twelfth day cannot be compared with the findings of Tammann *et al.* (1) since our analyses do not cover the same stages of healing. Working with healing skin transplants, they found that water increased to a maximum the first day then decreased as healing progressed but they did not carry their experiments beyond the fifth day.

The protein content of repair tissue reflects the sex difference found in the skin because, in every stage studied, males have a higher value than females. However, this sex difference in repair tissue is not statistically significant. The definite rise in the protein content of repair tissue occurring by the eighteenth day seems to be the result of a concomitant loss of water, because calculation of the protein content on the basis of dry weight does not show this increase. Tammann *et al.* (1) found no change in nitrogen content of skin transplants as healing progressed. However, their data were collected over such short periods of time (4–5 days) that it is not possible to compare their work with ours. Our own data show little difference in protein content at these early stages of healing.

SUMMARY

1. The water and protein content of skin and of repair tissue at periods of 3, 6, 9, 12, and 18 days after wounding were determined in young and old rats.

2. The water content of skin was found to be 60.1 $\pm$ 3.1% for males and 54.2 $\pm$ 3.6% for females. The water content of skin was not significantly different at the ages studied (108 days as compared with 400–600 days).

The protein content of skin was found to be 25.5 $\pm$ 2.2% for males and 22.6 $\pm$ 1.5% for females. The protein content of the skin was not significantly different at the ages studied.

3. The water content was much higher in repair tissue than in skin. From the third through the twelfth day, water content of repair tissue remained constant and was not influenced by age, sex or stage of healing. By the eighteenth day the water content had become lower and began to approach the value for skin.

4. The protein content was lower for repair tissue at the stages studied than for normal skin. It gradually increased during the 3, 6, and 9 day stage, coincidental with the most rapid healing. By the eighteenth day it had increased in the repair tissue to a value approaching that of skin. The protein content of repair tissue did not vary with sex or age.

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The Color Reaction of Certain Indoles and Imidazoles with Creatinine and Sodium 2,5-Dinitrobenzoate: Evidence for the Occurrence of Tautomeric Forms of Tryptophan and of Histidine

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INTRODUCTION

As part of a plan to compare certain properties of the 6 isomeric dinitrobenzoic acids, the rare 2,5-dinitrobenzoic acid was prepared in quantity several years ago (1) and the color reactions which it undergoes with creatinine and with reducing sugars were studied. Until recently there have been few conclusive results of general interest, and these have not hitherto been published. When creatinine and 2,5-dinitrobenzoate are allowed to react as in the 3,5-dinitrobenzoate method for the estimation of creatinine (2), the brown color which is formed has unsatisfactory characteristics and is of such great instability that there seems to be no good reason for the 2,5-dinitrobenzoate being used as a reagent for the estimation of creatinine.

However, the color which is given by urine with 2,5-dinitrobenzoate does not correspond with that which should be expected from the creatinine content of the urine, but is a much more intense red. In order to match the color given by 1 ml. of urine, it is necessary to use creatinine standards containing 5–10 mg./ml., and, even with these amounts, the colors of the standards are obviously different from those given by urine. One would, therefore, suspect the presence in urine of certain substances other than creatnine which react with 2,5-dinitrobenzoate but not with 3,5-dinitrobenzoate.

Tests with about 20 of the suspected components of urine showed that the important interfering substances give no color with 2,5-dinitrobenzoate except in the presence of creatinine. This may be demonstrated after precipitating the creatinine out of

urine as potassium creatinine picrate, and removing the picric acid color from the filtrate by extraction with ether. The remaining aqueous solution gives no appreciable color with 2,5-dinitrobenzoate until creatinine is added again, whereupon additional color is formed corresponding closely in depth to that which had been given originally by the urine. The substances are therefore considered to augment the creatinine 2,5-dinitrobenzoate color, and are thought to form complexes with the less deeply colored compound. The augmenting substances were found to be mainly histidine and tryptophan. Uric acid accounted for about 5% of the augmentation color, and other substances, such as arginine and tyrosine, which augment the color slightly, were shown to have a negligible effect in normal urine.

By analyzing urine for creatinine, histidine, and tryptophan (data unpublished), then making a standard containing the three substances in the proportions found, the color obtained matched closely that given by the urine with 2,5-dinitrobenzoate. It is considered, therefore, that histidine and tryptophan are responsible for approximately 95% of the augmentation color of normal urine. Study of these substances was undertaken, and in Section I of this paper are described the colors obtained with tryptophan and related indoles, and in Section II the colors with histidine and related imidazoles.

I. COLORS WITH INDOLES

The colors obtained with creatinine and 2,5-dinitrobenzoate in the presence of tryptophan seemed for a time not to be reproducible, being upon some occasions paler, and at other times darker, than was expected. This variation could not be attributed to the effects of air or of light, to the alkalinity of the solution, or to the order of mixing of the reagents. Whereas the intensity of color was known to depend upon the amounts of creatinine and of dinitrobenzoate used, its erratic development was unrelated to these substances. After somewhat prolonged study, the colors were found to depend upon the acidity or age of the tryptophan solution before it was added to the other reagents, and the variations in color were finally attributed to the existence in solution of 2 different structural forms of tryptophan. It is now believed that these forms are in tautomeric equilibrium, and that the colors which have been observed are determined by the relative amount of each form present. Data are presented herein to substantiate these conclusions. Indole and certain substituted indoles such as skatole and gramine give color which is not variable with the pH of the solution used, whereas indoleacetic acid and indican fail to alter the creati-

nine 2,5-dinitrobenzoate color at all. Thiamine, which contains the —N=C—linkage thought necessary for color production gives intense

$$\begin{array}{c} | \end{array}$$

color which also is unaffected by pH change of the thiamine solution.

EXPERIMENTAL

2,5-Dinitrobenzoic acid was prepared in this laboratory (1), a 6% solution of the sodium salt (2) being used. L- and DL-Tryptophan (Pfanstiehl, and Eastman Kodak) were purchased, and were not purified, as all preparations gave the same color values. Gramine and indoleacetic acid (Winthrop) and creatinine (Eastman Kodak) were also used as purchased. Solutions containing 1 mg. of creatinine/ml. and of 0.10 mg. of tryptophan (or other chromogen)/ml., both in 0.1 N HCl, were used as indicated subsequently.

Development and Measurement of Color

A reagent consisting of 20 ml. of 6% sodium 2,5-dinitrobenzoate, 10 ml. of creatinine solution, and 5 ml. of water was prepared as needed. It was stable over several days. For development of color (except for the data of Cols. VI and VII of Table I) 3.5 ml.

TABLE I

Color of Tryptophan with Creatinine 2,5-Dinitrobenzoate as Affected by Alkalinity of the Tryptophan Solution

Specific extinction coefficient $\times 10^{-3}$

Wave length	I 0.2 N HCl 3°C. for 4 days	II 0.1 N HCl freshly made	III 0.1 N HCl 9 days room temp.	IV pH = 2.4	V in NaHCO₃ 0.1 M	VI pH = 13 app.	VII Control -log T
500	21	15.6	62	30	58	85	.82
550	32	25	75	39	71	112	.32
600	22	19.3	49	30	43	71	.12

of this reagent was pipetted into a dry test tube, followed by 0.5 ml. of chromogen and 1.0 ml. of 5% NaOH.[1] This is Procedure 1. After 10 minutes the color was measured by use of a Beckman spectrophotometer, cells 1 cm. thick being used. For the control color 0.5 ml. of water was used in place of the chromogen.

To establish comparable colors, the concentrations of reagents during the color development were kept constant. To minimize undesired tautomeric change of chromogen, the time allowed for pipetting and for almost instant mixing of solutions was not varied. Only values obtained with 0.05 mg. of chromogen in the presence of 1 mg. of creatinine (ratio = 1:20) are reported. E values are specific extinction co-

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[1] Variations of ±0.2 ml. of 5% NaOH altered the final color only in proportion to the volume difference: for this reason, the pH of the colored solutions was not determined, and water, instead of 0.1 N HCl, was used in the control color.

efficients, measured at wave lengths 500, 550, and 600 mμ, and calculated according to the equation

$$-\log T = K \times 0.01 \times 1.0.$$

0.01 = mg. tryptophan/ml. of colored solution.

1.0 = thickness of cell in cm.

T = transmission by the tryptophan solution measured against reagents without tryptophan.

K = specific extinction coefficient.

RESULTS

The colors obtained with tryptophan are given in Table I. The solutions, although identical in composition in each case, varied from brown to deep red depending upon the treatment accorded the tryptophan before it was mixed with the creatinine-dinitrobenzoate reagent. A freshly prepared solution of tryptophan gave the extinction values of Col. II, and as it aged at room temperature, the color increased over several days to give the equilibrium values of Col. III. When the fresh tryptophan solution was mixed with a buffer or with 0.2 M $NaHCO_3$, a transformation of the tryptophan occurred immediately, the values of Cols. IV, at pH 2.4, and V, in 0.1 M $NaHCO_3$, being obtained. The pH values were measured with a Beckman pH meter (glass electrode). For the figures of Col. VI, 1.0 ml. of tryptophan solution was mixed with 2.0 ml. of 5% NaOH, and 1.5 ml. of the resulting solution was pipetted into the 3.5 ml. of reagent for color development. Thus the alkali and the tryptophan were added simultaneously to the creatinine-dinitro-benzoate reagent. This is Procedure 2.

Further evidence that the colors given by tryptophan and 2,5-dinitrobenzoate depend upon an equilibrium between 2 forms of tryptophan was obtained by heating a solution of tryptophan in 0.1 N $NaHCO_3$ and analyzing the solution at intervals. The color values before heating corresponded with those in Col. V of Table I, whereas after refluxing for one hour they corresponded with those of Col. VI. While standing at room temperature overnight in a stoppered flask the color reverted again to its former values (Col. V). The cycle of change was repeated by reheating the solution. Tryptophan, upon being heated in 0.2 N HCl, or in well buffered solution at pH 8 for 5 hours did not show corresponding changes.

DISCUSSION

The color variations shown in Table I must be attributed to the tryptophan solution alone since all other components and the order of their mixing were constant. Tryptophan is known to withstand much

more concentrated acid or alkali than was used (4, 5, 6, 7). That the amount of chromogen present was at all times equivalent to the amount of tryptophan originally used was repeatedly determined by Procedure 2, the color of Col. VI being constantly reproduced.

Five possible causes for the color differences have been considered: (a) opening and closing of the indole ring, (b) optical isomerism, (c) order of arrangement of groups in a complex, whether in the order A-B-C, A-C-B, or B-A-C, (d) addition of one or several molecules of tryptophan to form a complex, (e) existence of tautomeric forms of tryptophan in a complex. The hypothesis that the color differences may be attributed to the existence of two tautomeric forms of tryptophan is consistent with the properties of tryptophan and with the facts which are described herein.

The two forms of tryptophan postulated are:
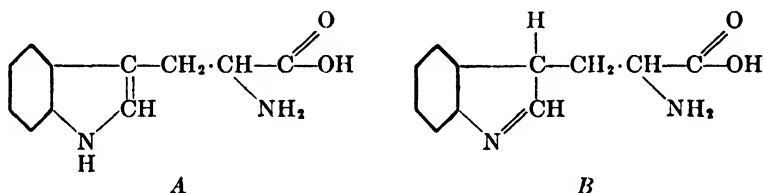
Judging from the color variations with freshly made solutions of trypto-phan, equilibrium between the two forms in acid solution seems to be established only after several days, whereas in alkali, less than one minute is required for pipetting and mixing, and the equilibrium seems to be reached in that time. The form of tryptophan giving the deeper color is the one more stable in alkali, and by analogy with the known tautomerism of pyrrole compounds (3), is assumed to have the structure B. Both of the tautomers must add either to the colorless form of creatinine 2,5-dinitrobenzoate, or to one of its components, at a rate faster than that of the tautomeric change: otherwise there would be but one intensity of color observed. The gradual increase of color with tryptophan in 0.1 N HCl over several days has been observed re-peatedly, and was at first thought to indicate either a third form of tryptophan, or that some other equilibrium than a tautomeric change was involved. However, the colors given by tryptophan under different conditions differed quantitatively but not qualitatively. It seems therefore, that one equilibrium only is involved.

In order to test the hypothesis that the structure "B" applies to the "red" form of tryptophan, tests were made of the augmentation by L-proline and thiamine. Proline contains a reduced pyrrole ring and was expected to show no augmenting effect. This assumption was confirmed. Thiamine contains the —N=C— grouping,   postulated for structure "B," and, in the absence of labile hydrogen in the thiazole ring, only one intensity of color was anticipated. This likewise was confirmed. Skatole, indole, and gramine, in both acid and alkali, likewise gave only one intensity of color. From the fact that indoleacetic acid, 1,3-dimethylindole, and indican gave no augmentation color, it is evident that the nature of the substituent group attached to the indole ring determines the ability of the compound to produce color.

Data for the above substances are shown in Table II.

TABLE II

Color of Several Indole Compounds and of Thiamine with Creatinine and 2,5-Dinitrobenzoate[a]

Specific extinction coefficients $\times$ 10^{-3}

Wave length	Gramine[b]	Indole	Skatole	Thiamine
500	32	17	31	34
550	17	8	17	21
600	9	3.8	8.4	10

[a] In each case the same intensity of color was obtained from acid and alkaline solution of the substance. Freshly prepared solutions in 0.1 N HCl were used.

[b]
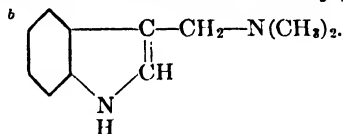
Proteins such as casein and egg albumin give augmentation colors with creatinine 2,5-dinitrobenzoate which seem to be proportional to their tryptophan contents. Preliminary observations indicate that 5 mg. of egg albumin and 3 mg. of casein, which both contain about the same amount of tryptophan, estimated from tables of composition of proteins, give closely comparable colors, which are the same by Procedures 1 and 2. More exhaustive study of the color reaction should be made, however, before conclusions are drawn regarding the structure of tryptophan in proteins.

Evidence for tautomerism of tryptophan has been sought in the literature. Many of the methods for the analysis of tryptophan require the use of strong acid solutions,

in which tryptophan would exist mainly in one form. In the Folin-Looney (8) procedure for tyrosine and tryptophan, the latter substance gives more color after treatment with mercury than before, and the standard is routinely carried through a mercury precipitation procedure to intensify its color. It is not improbable that the formation of one tautomer is favored by this treatment. The absorption in the ultraviolet by tryptophan has been shown by Holiday (9) to be displaced as the medium is changed from 0.1 N HCl to 0.1 N NaOH, although the slight displacement of the curve was not considered to represent a structural rearrangement in the indole ring.

II. Colors with Imidazoles

The effect of histidine of urine in augmenting the color given by creatinine and 2,5-dinitrobenzoate in alkaline solution has been described in the introduction to this article. In attempts to estimate the histidine content of solutions by measurement of the color, it was discovered that histidine in acid gives somewhat different intensity of color from that by histidine in alkali, just as was found for tryptophan. It was suspected that histidine, and perhaps other imidazole compounds, might exist in tautomeric forms, and that the color of a complex containing one form would differ from that containing the other. Two modifications of histidine which might exist in equilibrium are C and D:
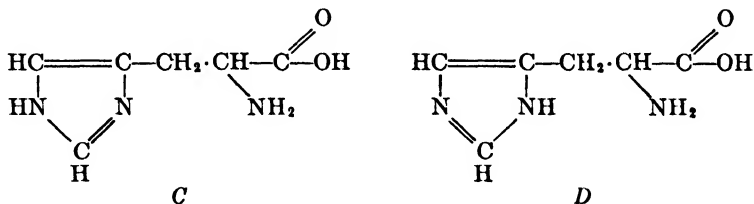
It would seem reasonable to expect that the acidic properties of the ring nitrogen atoms would differ because of their respective distances from the side chain, and also because of the influence of the unsaturated group —HC=C— which is interposed between the side chain and one atom of nitrogen, but not the other. It would be reasonable, therefore, to expect the two forms to exist in different relative amounts in acid and alkaline solutions. Search of the literature, however, has revealed no experimental data which distinguish tautomeric forms of histidine, or of any imidazole compound.

EXPERIMENTAL

L-Histidine and histamine were used as purchased. Carnosine was prepared as the copper salt in this laboratory. Imidazole and hydroxymethylimidazole were supplied by Dr. G. J. Cox, and anserine copper salt by Dr. Wm. A. Wolff.

The procedures for developing and measuring the colors were exactly analogous to those used in the study of indoles (Section I). Readings of color were started within seconds after 10 minutes had been allowed for color development, they were made in systematic order, and were completed in less than 2 minutes. Frequent repetition of the determinations established that the experimental errors were considerably smaller than the differences observed for histidine dissolved in acid and alkali. Specific extinction coefficients are shown in Table III.

TABLE III

Color of Several Imidazole Compounds with Creatinine and 2,5-Dinitrobenzoate
Specific extinction coefficients $\times 10^{-3}$

Wave length	Histidine			Histamine	Carnosine	Hydroxy-methyl-imidazole	Imidazole
	(I) Fresh	(II) Equil.	(III) Alkaline	(IV)	(V)	(VI)	(VII)
500	53	40	57	43	30	38	15.3
550	29	21	36	19	11.7	12	8.2
600	16	11	20	8	6	6.4	5.2

Histidine in 0.1 N HCl, Col. I, gives a deeper color after a few minutes than after an hour, Col. II. Both solutions give increased color after being made alkaline, Col. III. Among the imidazoles, this behavior was observed only with histidine. Evidently the nature of the side chain influences some property of the imidazole ring, which we interpret to be a tautomeric change. It is noteworthy that, among all the indoles and imidazoles studied, this change has been found only in the amino acids. The amphoteric nature of the side chain may be the important factor which alters the equilibrium in the ring and makes possible the formation of two complexes. Anserine gives only a trace of augmentation color, for which carnosine as impurity may be responsible. It is at once evident that the methyl group in place of the imide hydrogen of the imidazole ring greatly inhibits, or completely prevents, the color formation, which, therefore, must involve the presence of an imide hydrogen.

SUMMARY

1. Among the indole compounds studied, indole, skatole, gramine, and tryptophan increase the color developed between creatinine and 2,5-dinitrobenzoate. Indoleacetic acid and indican do not do so.

2. Among the imidazole compounds studied, imidazole, hydroxymethylimidazole, histidine, histamine, and carnosine increase, whereas anserine fails to increase, the color as in 1.

3. The amount of increase of color produced by tryptophan and histidine seems to be a function of the pH of the tryptophan or histidine solution used. The color given by the other substances does not vary with pH.

4. The change undergone by tryptophan and histidine with changing pH seems to correspond in each case to altering a tautomeric equilibrium between two forms of the molecule.

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Concentrations of Ribonucleic Acid and Desoxyribonucleic Acid in Animal Tissues

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INTRODUCTION

The knowledge of the concentrations of ribo- and desoxyribonucleic acids is a fundamental requirement for the elucidation of the role which nucleoproteins play in cellular life, in mitosis, and in protein synthesis. Several attempts have, therefore, been made, using different methods, to determine these concentrations in different normal and abnormal animal tissues. Among the color reactions used for quantitative purposes, that with diphenylamine, described by Dische (1), has hitherto almost exclusively been applied for the determination of desoxyribonucleic acid and for nucleotides and nucleosides containing desoxyribose. However, it is known, that the Dische reaction is also given by compounds other than desoxyribose and its derivates (2).

The applicability of the diphenylamine test to the estimation of desoxyribonucleic acid (DNA) in various materials is further limited by the fact that many compounds, which do not give Dische coloration under the usual conditions for this test, influence the color intensity obtained with pure DNA (3). In this connection the great sensitivity of the Dische test to the presence of inorganic salts and proteins should primarily be mentioned. Not only the diphenylamine test, but also other color reactions thus far used for estimation of DNA are more or less disturbed by the presence of protein.

Accurate quantitative estimations of the DNA content of animal tissues by the colorimetric methods available at the present time cannot be made without at least a partial separation of the acid from other tissue constituents.

A method for the isolation of nucleic acids from animal tissues has recently been devised by Davidson and Waymouth (4). This method involves extraction of the dry

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tissue powder with 10% sodium chloride solution and precipitation of the extracted nucleic acids as lanthanum salts. However, desoxyribonucleic acid is not completely extracted from the tissue cell nuclei by this procedure. Some losses of nucleic acids may occur during the precipitation and decomposition of the lanthanum salts.

Another procedure for the elimination of tissue constituents interfering with the determination of ribose and desoxyribose nucleic acid was recommended by Schneider (5). This procedure consists in removal of acid-soluble phosphorus compounds and phospholipides, and in subsequent hydrolysis of the tissue residue with hot trichloroacetic acid.

The methods at present available were reexamined by the authors (6). A new color test for ribonucleic acid (RNA) was evolved, which is not disturbed by desoxyribose and moderate amounts of proteins. Furthermore, modifications of the Davidson-Waymouth procedure were described (6, 7), giving quantitative yields of RNA and DNA. Our present values for RNA and DNA have been obtained by the following methods.

METHODS

Extraction of Nucleic Acids from Tissues

For the extraction of ribonucleic acid from animal tissues a procedure was proposed by the authors (6) consisting in treatment with 10% sodium chloride solution followed by treatment with cold 0.2 N NaOH. The use of a stronger alkali or of higher temperatures must be avoided because of the sensitivity of ribonucleic acid to alkali. On the other hand, desoxyribonucleic acid was only incompletely extracted by the above procedure (7). Treatment with hot 0.5 N sodium hydroxide is necessary to obtain quantitative yields of tissue desoxyribonucleic acid.

For determination of both ribonucleic acid and desoxyribonucleic acid in animal tissue the latter must be homogenized, one fraction being extracted according to the procedure suitable for ribonucleic acid and another according to the more drastic method yielding a quantitative extraction of desoxyribonucleic acid.

Procedure for Determining Ribonucleic Acid

One g. of the finely minced tissue is ground with sand or glass powder in a mortar with addition of 10 ml. 10% sodium chloride solution in small portions. After 2 hours standing at 0°C., the mixture is centrifuged. The residue is extracted once more as described above. The extraction procedure is then repeated twice using 0.2 N NaOH as solvent. The volume of the combined extracts is made up to 40 ml. 25 ml. of this solution is measured into a centrifuge tube. The pH is adjusted to about 8 with acetic acid and the volume is made up with distilled water to 33 ml. Two per cent lanthanum acetate and 50 ml. absolute alcohol are added. After keeping the mixture in the refrigerator for 1 hour or more, the precipitate of lanthanum nucleate is centrifuged, washed twice with 10 ml. 0.1% lanthanum acetate [1] in 50% alcohol, and decomposed

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[1] In spite of the low temperature in the refrigerator a small part of the RNA could be broken down by the action of nucleases. In view of the fact that even lower nucleotides are precipitated by lanthanum acetate and thus enclosed in our procedure for determining RNA, and in view of the low activity of nucleases at 0°C., this error must be considered negligible.

with 1.2 ml. 0.5 M sodium carbonate solution. After dilution with 4.8 ml. distilled water the mixture is centrifuged and the residue washed twice with 3 ml. 0.1 M sodium carbonate solution. The volume of the combined solutions is made up to 12 ml.

The determination of ribonucleic acid in the extract is carried out using the phloroglucinol color reaction described earlier (6).

Procedure for Determining Desoxyribonucleic Acid

One g. of the finely minced tissue is ground with glass powder in a mortar, 10 ml. 0.5 N NaOH being added in small portions. The mixture is then kept at room temperature for at least 1 hour and then heated in a boiling water bath for 1 hour. After centrifuging, the undissolved residue is treated with 7.5 ml. 0.5 N NaOH for 0.5 hour in the water bath. This latter procedure is then repeated once more. The combined extracts are neutralized with acetic acid (pH 7.5–8.0) and the volume made up to 30 ml. The nucleic acids are precipitated and purified as described for ribonucleic acid. The determination of the desoxyribonucleic acid is carried out according to Dische (1).

RESULTS

The ribo- and desoxyribonucleic acid content of tissues of normal and cancerous rats is seen from Tables I–III.

As mentioned in earlier papers (3), the Dische test gives values which are too high in the presence of proteins. The nucleic acid fraction obtained by lanthanum precipitation contains appreciable amounts of proteins and we expect the Dische values, obtained in such fractions, to be approximately 15% too high. Furthermore, we must take into account that our standard nucleic acid preparations cannot be regarded as pure compounds, the desoxyribonucleic acid showing a higher degree of purity than the ribonucleic acid.[2] Consequently the values given in Tables I–III for the ratio RNA/DNA must be denoted as the lower limit, the actual value (average corr.) being about 30% higher.

DISCUSSION

The absolute values for the concentrations of RNA and DNA in rat livers, as determined by different authors and by different methods, show great variations. This may be partially caused by the fact that the nucleic acid content of organs is remarkably dependent on the age and the diet (8) of the experimental animals. However, the lack of agreement depends to a great extent on differences in the methods.

[2] The P content of our DNA standard amounted to 9.38%, while the standard RNA contained 8.11% P.

One purpose of this investigation was to obtain exact values for the *ratio RNA/DNA*, these values being of extreme importance for the understanding of nucleic acid metabolism. This ratio is generally high in rapidly growing organs in which protein synthesis is vigorous. The important question, namely, to what extent this ratio may be the result of a reversible conversion of RNA and DNA on living cells, cannot yet be answered.

For normal rat *liver* Schmidt and Thannhauser (14) found by their method the ratio RNA/DNA = 3.9. According to earlier experiments, carried out at this institute (Hahn; Solodkowska; Řeřábek), the average value was 3.1. Using modifications of our previous colorimetric

TABLE I

Nucleic Acid Content of Liver

Number of rat	Weight of rat g.	Ribonucleic acid content		Desoxyribonucleic acid content		Ratio RNA/DNA
		mg./g. fresh weight	mg./g. dry weight	mg./g. fresh weight	mg./g. dry weight	
A. Normal Rats						
Rn 1	145	8.00	27.0	4.50	15.2	1.78
Rn 2	157	6.90	23.3	4.40	14.9	1.57
Rn 4	200	7.64	26.2	4.20	14.3	1.82
Rn 5	210	6.89	23.3	4.20	13.6	1.74
Rn 7	122	8.15	27.5	4.10	13.9	1.99
Average		7.52	25.5	4.24	14.4	1.78
				Average corr.		2.4
B. Rats with Jensen Sarcoma						
Rs 4	200	7.40	29.0	4.17	16.3	1.82
Rs 5	145	9.70	35.6	3.36	12.3	2.89
Rs 6	130	8.65	33.8	3.69	14.4	2.35
Rs 7	120	8.13	29.3	3.96	14.3	2.06
Rs 8	140	9.63	36.1	3.80	14.2	2.53
Rs 9	175	7.13	25.4	3.75	13.4	1.90
Rs 10	130	6.29	22.2	3.76	13.3	1.68
Rs 11	145	7.82	31.4	2.66	14.6	2.14
Rs 12	105	7.93	33.2	3.22	14.0	2.44
Average		8.07	30.6	3.72	14.1	2.17
				Average corr.		2.8

TABLE II

Nucleic Acid Content of Spleen

Number of rat	Ribonucleic acid content		Desoxyribonucleic acid content		Ratio RNA/DNA
	mg./g. fresh weight	mg./g. dry weight	mg./g. fresh weight	mg./g. dry weight	
A. Normal Rats					
Rn 4–5	5.72	23.0	13.4	5.48	0.43
Rn 6–7	5.69	25.9	14.6	66.6	0.39
Average	5.71	24.0	14.0	60.7	0.41
			Average corr.		0.5
B. Rats with Jensen Sarcoma					
Rs 1–3	6.03	26.0	13.4	58.0	0.43
Rs 4–6	7.36	33.8	14.8	66.5	0.50
Rs 7–8	6.60	29.1	13.9	61.5	0.48
Rs 10	5.24	22.6	16.5	71.2	0.32
Rs 11–12	5.46	26.2	14.3	69.0	0.38
Average	6.14	27.5	14.6	65.2	0.42
			Average corr.		0.5

TABLE III

Nucleic Acid Content of Heart and Brain

Number of rat	Ribonucleic acid content		Desoxyribonucleic acid content		Ratio RNA/DNA
	mg./g. fresh weight	mg./g. dry weight	mg./g. fresh weight	mg./g. dry weight	
Heart					
Rn 8–10 norm.	3.14	12.4	3.06	12.1	1.03
Rs 1–3 sarc.	4.50	19.5	4.10	16.8	1.10
Rs 4–6 sarc.	3.63	16.2	2.70	12.1	1.35
Rs 7–8 sarc.	—	—	2.65	11.1	—
Brain					
Rs 13–15 sarc.	1.96	9.3	1.70	8.1	1.15
Rs 16–17 sarc.	2.37	11.3	19.5	9.3	1.21

TABLE IV

Ratio RNA/DNA

Normal tissues	Davidson and Way-mouth (4)	Schmidt and Thann-hauser (14)	Walter C. Schneider (5)	Jar. Refábek (13)	Solodkowska and v. Euler	v. Euler and L. Hahn		
						(6)	(F)	(F) corr.
1	2	3	4	5	6	7	8	9
Brain	2.1	2.2	1.4	1.5	1.8	—	1.2	1.6
Heart	3.6	—	—	1.2	—	—	1.1	1.5
Intestinal mucosa	—	—	—	—	—	0.6	—	
Kidney	1.8	1.4	—	1.1	—	0.5	—	
Liver	3.5	3.9	3.5	3.1	3.1	—	1.8	2.4
Lung	0.8	—	0.4	—	0.5	0.6		
Bone marrow	—	—	—	—	0.8	—		
Muscle	3.6	—	—	2.1	—	2.3		
Pancreas	8.0	15.4	—	—	—	—		
Spleen	0.6	1.3	—	0.8	—	0.7		
Thymus	0.2	0.3	—	—	—	—		

method and introducing the corrections for the influence of proteins and for our standard nucleic acid preparations, we find for the liver of normal rat, 5–11 months of age, the average ratio 2.4.

Organs of Jensen Sarcoma-bearing rats

The nucleic acid content of cancerous organs was compared earlier by several authors with that of normal organs and with adjacent tissues (9, 10,11). Stowell found in 18 of 20 tumors the amount of DNA, per unit volume and per cell, greater than in the adjacent homologous normal tissues. Two-thirds of the tumors had more RNA per unit volume and per cell than the corresponding normal tissue, and in half of these the increase was more than 50%. J. E. Davidson and C. Way-mouth (12) found that the ratio RNA/DNA (measured by the P values) is lower in liver tumors and in embryo livers than in the normal liver, although in both the liver tumor, and in the embryo, the concentrations of RNA and DNA are higher than in normal liver.

In the livers of our Jensen sarcoma-bearing rats no metastases could be macroscopically detected. In these livers the ratio RNA/DNA = 2.8; it is somewhat but not significantly higher than for the liver of normal rats (2.4). We find, however, the difference between the

RNA/DNA ratio in normal and in sarcoma-bearing rats smaller than it was found by Řeřábek whose results were 3.1 and 4.3, respectively (13).

In heart and brain (no distinction was made between different brain regions) of sarcoma-bearing rats, the ratio RNA/DNA was not higher than that of normal rats.

Summary

The content of ribonucleic acid (RNA) and of desoxyribonucleic acid (DNA) in liver, spleen and heart of normal and cancerous rats was determined.

The ratio RNA/DNA was found for:

Organ	Normal rats	Sarcoma-bearing rats
Liver	2.4	2.8
Spleen	0.5	0.5
Heart	1.5	1.6

The difference of the ratios in liver, though distinct, cannot be regarded as significant.

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Yeast Nucleic Acid. I. Quantitative Determination by Spectrophotometry; Ammonium and Phosphate Ions as Factors in Biosynthesis

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INTRODUCTION

A literature survey revealed that methods for the estimation of nucleic acids in animal tissues are numerous, but that few methods have been applied specifically to yeast.

Decker and Dirr (1) reported that non-nucleic acid purines can be extracted from yeast with 10% trichloroacetic acid at room temperature. By analyzing a yeast for its total purines and the trichloroacetic acid extracts for acid-soluble purines, these workers determined nucleic acid purines by difference. Then, converting nucleic acid purine values to nucleic acid values, they found that six yeasts contained from 2.42 to 6.33% nucleic acid (dry basis). In this laboratory, the Decker and Dirr purine method was applied to a large number of yeast samples, but it was regarded as unsatisfactory, mainly because it is very time-consuming. Other purine procedures have been criticized for requiring acid treatments capable of causing chemical losses of purines (2, 3, 4).

Jeener and Brachet (5) correctly commented that the determination of nucleic acid by purine analyses "requires hours of delicate work." They therefore devised a procedure for analysis of nucleic acid, which was dependent upon the basophilic property of nucleic acid. This procedure was more direct. It did not require the separation of nucleic acid from yeast. However, in this laboratory, it was found that the basophilic type of nucleic acid analysis did not give reliable and reproducible results.

Caspersson and Brandt (6) published a spectrophotometric technique for determination of nucleic acid in yeast. At best, this method permits only approximations of the nucleic acid present in the yeast, since materials such as proteins, purines, and pyrimidines, which interfere with the analysis, are not removed.

It was reported by Wiame (7, 8) that a metaphosphate is formed and accumulated in yeast. He demonstrated that this metaphosphate possesses solubility properties very similar to those of nucleic acids. Consequently, Schmidt et al. (9) stated that "the determination of nucleic acid phosphorus should be supplemented by ribose or purine determinations."

During the investigation herein reported, the problem of obtaining a satisfactory analytical method for determining yeast nucleic acid was attacked by adapting the ribose method of Mejbaum (10) to yeast, by developing a spectrophotometric method, and by comparing these two methods with the purine method. No attempt was made at this time to differentiate between ribonucleic acid and desoxyribonucleic acid since the latter is reported to contribute very slightly to the total nucleic acid content of yeast (5). The effects of ammonium and phosphate ions upon the biosynthesis of nucleic acid by yeast were also studied, and the nucleic acid contents of yeast cells were determined.

EXPERIMENTAL

Yeast was extracted with sodium hydroxide solution under conditions employed in commercial practice for nucleic acid isolation (11). The pale yellow solution so obtained was observed to show an absorption peak at about 2600 Å. and also to follow Beer's law. The ultraviolet absorption curve is Curve 3 in Fig. 1. It was realized that such a solution contained not only nucleic acid, but many water-soluble compounds including purines, pyrimidines, nucleosides, nucleotides, vitamins, *etc.*
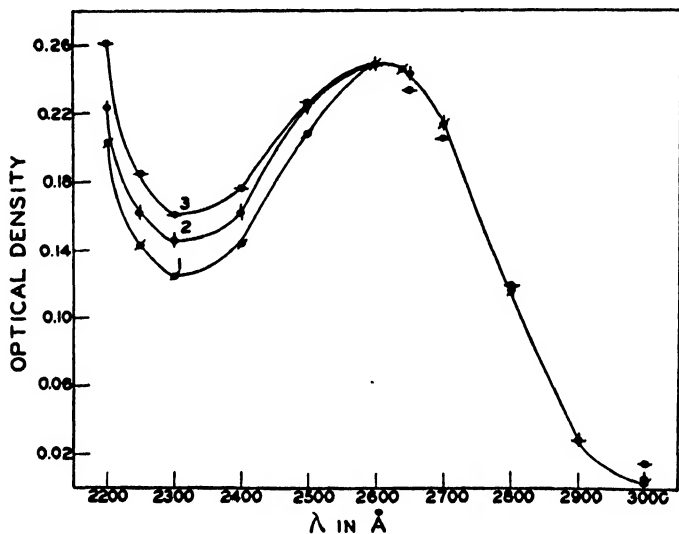
FIG. 1. Effect of trichloroacetic acid treatment on the ultraviolet absorption spectrum of NaOH yeast extracts. 1. NaOH solution of Eimer and Amend's purified yeast nucleic acid. 2. NaOH extract (or hot trichloroacetic acid extract) of yeast previously treated with trichloroacetic acid. 3. NaOH extract of untreated yeast.

Yeast extracted with trichloroacetic acid as prescribed by Decker and Dirr for the removal of acid-soluble compounds was treated in 2 ways. One-half was extracted with cold alkali as in isolation (11), and the other with hot trichloroacetic acid solution as in the method of ribose analysis (12). In view of the specificity associated with these extractions, the finding that both solutions displayed the same ultraviolet absorption spectrum (Curve 2, Fig. 1) was accepted as significant.

Comparison of Curves 3 and 2 with Curve 1, which was prepared from purified yeast nucleic acid, indicated that the preliminary trichloroacetic acid extractions effected extensive purification.
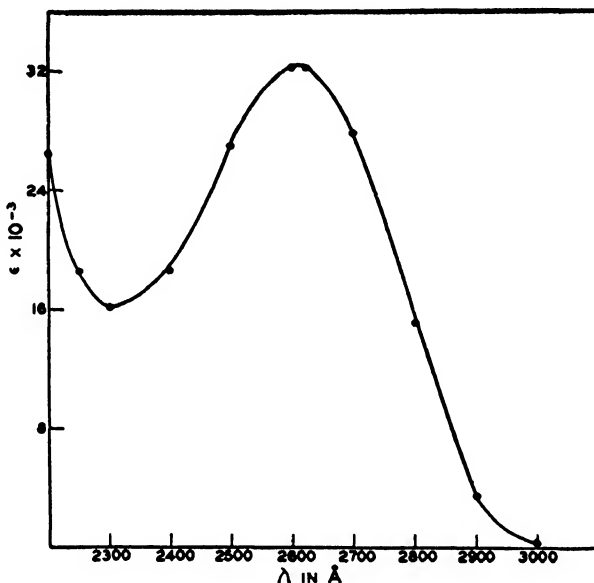
Fig. 2. Ultraviolet absorption of yeast nucleic acid in 0.01 N NaOH solution.

Samples of purified yeast nucleic acid from Eimer and Amend, Eastman Kodak, Schwarz Laboratories, Pfanstiehl Chemical Co., Distillers Co., Ltd., and Standard Brands were dried *in vacuo* and their ultraviolet absorption spectra were determined using 0.01 N sodium hydroxide as the solvent. The differences were slight. The curve obtained in this manner from Eimer and Amend nucleic acid is shown in Fig. 2. It is in excellent agreement with curves published by Sisters Stimson and Reuter (13).

A series of solutions of nucleic acid at different concentrations were prepared in 0.01 N sodium hydroxide. Optical density readings were taken in 1 cm. quartz cells with the Beckman spectrophotometer set at 2600 Å. The curve so obtained (Fig. 3) serves as the standard curve for the spectrophotometric method which was developed and which is described in detail below.

Methods for Determination of Nucleic Acid in Yeast

Spectrophotometric Method. A 10.00 g. sample of pressed yeast is stirred for 15 minutes with 30 ml. of 10% trichloroacetic acid solution at room temperature. The mixture is centrifuged at 4000–5000 r.p.m. for 5 minutes and the supernatant liquid is discarded. Three such extractions are performed and are followed by extraction with 20 ml. of water in the same manner.

The residue is treated in either of two ways:

1. It is stirred with 20 ml. of 2.2% sodium hydroxide solution at 10–15°C. for 1 hour. The pH is adjusted to 6.5–6.7 and the mixture is centrifuged. The residue is twice washed by stirring for 10 minutes with 20 ml. volumes of water. Then aliquots
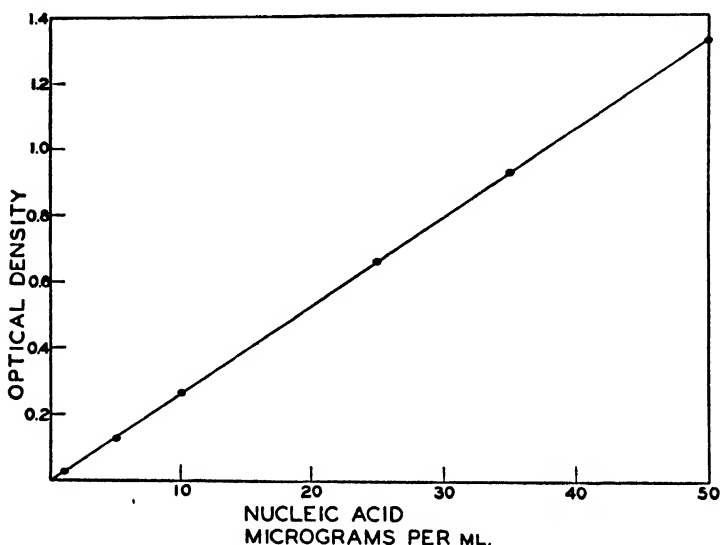
Fig. 3. Standard curve for the spectrophotometric method for the determination of nucleic acid in yeast.

of the combined sodium hydroxide and aqueous extracts are appropriately diluted with 0.01 N sodium hydroxide. Optical density readings are taken at 2600 Å. and calculations of the nucleic acid content of the yeast are made by application of the standard curve (Fig. 3).

2. It is stirred for 15 minutes with 30 ml. of 5% trichloroacetic acid at 90°C. The residue is washed at room temperature by brief stirring, first with 15 ml. of 5% trichloroacetic acid, and then with 30 ml. of water. Aliquots of the combined extracts are diluted with 0.01 N sodium hydroxide. Optical density readings are taken and nucleic acid estimated as under "1" above. With the dilutions employed the absorption at 2600 Å. by trichloroacetic acid is negligible.

Adaptation of Ribose Method to Yeast. Mejbaum (10) devised a colorimetric technique by which ribose could be determined in organic compounds. This work has been extended (12) for the determination of nucleic acid in animal tissues. In this laboratory, the method was adapted to yeast usage.

Purine Method. The purine method employed for comparison with the above methods was that of Decker and Dirr improved in accordance with suggestions by Hitchings and Fiske (14).

RESULTS

Comparison of Nucleic Acid Determinations. Table I records the results of nucleic acid analyses on 5 yeasts by the purine method, the

TABLE I

Nucleic Acid Analyses by Different Methods on Various Yeast Samples

Yeast	Per cent Nucleic acid (calculated to dry basis)			
	Spectrophotometric		Purine method	Adapted ribose method
	NaOH extraction	Hydrolysis with Cl₃CCOOH		
Bakers' A	4.31	4.38		4.67
Bakers' B		3.68	3.58	3.82
Bakers' C	7.38	7.60		7.35
Brewers' A, primary	4.20	4.32	4.12	
Brewers' B, primary	3.16	3.28	3.37	5.05

adapted ribose method, and the two variations of the spectrophotometric method.

Effects of Ammonium and Phosphate Ions on Biosynthesis of Nucleic Acid by Yeast. Yeasts were cultivated on a pilot plant scale in media which differed only in their ammonium and phosphate ion concentrations. All of the final yeasts were propagated from the same seed yeast to the same growth stage. Contained in Table II are analyses of these yeasts for nitrogen and nucleic acid.

Nucleic Acid Content of Yeast Cells. By using a hemocytometer, the number of cells/g. of yeast was determined on 4 yeasts. The weights of nucleic acid per cell were calculated from nucleic acid analyses. The results are recorded in Table III.

TABLE II

Effect of Ammonium and Phosphate Ion Concentrations on the Nucleic Acid and Total Nitrogen Contents of Experimental Bakers' Yeasts

Ammonium ion con-centration in medium	Phosphate ion con-centration in medium	Yeast analyses (dry basis)	
		Total nitrogen	Nucleic acid
		Per cent	*Per cent*[a]
High	High	8.67	7.17
Medium	High	8.31	6.39
Low	High	6.68	4.28
Medium	Low	8.23	4.40
Low	Low	6.55	4.06

[a] Nucleic acid data in this and following table obtained by Cl_3CCOOH type spectrophotometric analysis.

TABLE III

Nucleic Acid Content of Individual Yeast Cells

Yeast	Nucleic acid in yeast (dry basis)	Yeast cells/g.	Mg. nucleic acid/ yeast cell
	Per cent		
Bakers' A, stage 1	7.52	41.9×10^9	1.79×10^{-9}
Bakers' A, stage 2	5.97	51.0×10^9	1.17×10^{-9}
Bakers' D, sample 1	3.56	46.2×10^9	0.77×10^{-9}
Bakers' D, sample 2	3.06	40.6×10^9	0.75×10^{-9}

DISCUSSION

The differences in Curves 2 and 3 (Fig. 1) below 2600 Å. are due to the removal of unidentified materials from yeast by extraction with 10% trichloroacetic acid at room temperature. The observation that these compounds have absorption curves which drop rapidly between 2200 Å. and 2600 Å. (coupled with their solubility in trichloroacetic acid, in alkali, and in water at pH 6.5–6.7) makes it possible that they consist mainly of amino acids and polypeptides. The fact that their absorption at 2600 Å. is very low, whereas that of nucleic acid is very high, permits good analytical results even in the presence of small quantities of these foreign materials

There was general agreement among the nucleic acid values obtained by the purine, ribose, and spectrophotometric methods, but

some differences were noted. When the spectrophotometric method was used, the sodium hydroxide type of extraction produced values slightly lower than those obtained by hydrolysis with hot trichloroacetic acid. The purine method values were generally lower than the corresponding spectrophotometric analyses, whereas the ribose method values were usually higher.

Duplicate spectrophotometric analyses fell into close agreement. This was not true for the other methods. In one instance, a fresh yeast was analyzed by the 3 methods and the analyses were repeated after storage of the yeast for 3 weeks in a refrigerator. The nucleic acid content as determined by spectrophotometry remained constant, but the purine results fell off sharply, and the ribose values increased.

The spectrophotometric procedure is preferred for the following reasons:

1. It is direct, whereas the other methods require conversions. (In the purine method, the nucleic acid is cleaved to purines which are precipitated as copper salts and then analyzed for nitrogen. The ribose determination is dependent upon the hydrolysis of nucleic acid to ribose, followed by conversion to furfural which is estimated after the formation of a blue complex with orcinol.)

2. It is reliable regardless of the condition or age of the sample.

3. It offers the best duplicability.

4. It is the fastest.

It should be mentioned that the analytical values presented in Table I were checked, in a general way, by isolation work.

Caspersson et al. (6, 15, 16, 17, 18, 19) have clearly established the generalization that a high concentration of nucleic acid is characteristic of the cytoplasm of cells in which rapid protein synthesis is taking place. A high concentration of nucleic acid in the cytoplasm of yeast is correlated with growth (6, 20). This parallelism between nucleic acid and protein contents is illustrated by the data in Table II, where it is evident that the percentage of nucleic acid in yeast cells can be regulated by cultivation under conditions favorable to the rapid or slow biosynthesis of proteins. Several other types of yeast propagated in media differing in ammonium and phosphate ion concentrations showed similar effects.

The concentration of nucleic acids (mainly ribonucleic acid) in yeast was found to be 0.75–1.79 $\times$ 10^{-9} mg./cell (Table III). Ris (21)

reported that calf thymus contains desoxyribonucleic acid to the extent of $1.1-1.2 \times 10^{-9}$ mg./nucleus.

ACKNOWLEDGMENT

SUMMARY

1. A spectrophotometric method for the quantitative determination of nucleic acid in yeast is described.

2. The effects of ammonium and phosphate ions upon the biosyntheses of nucleic acid and protein by yeast are shown.

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Displacement Analysis of Lipides. Preliminary Studies with Normal Saturated Fatty Acids

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INTRODUCTION

Natural fats contain a mixture of closely related fatty acids, the separation and measurement of which presents a difficult problem. Chromatography offers the possibility of such separation and measurement in small samples, and some attempts to apply chromatography to fatty acid analysis have been made. Cassidy (1) and Kaufman and Wolf (2) have made studies of elution analysis applied to fatty acids, and Claesson (3) has done a great deal of work using frontal analysis. These methods do not give complete separations, but displacement analysis (4) offers the possibility of simultaneous measurement and separation of the components of mixtures of members of homologous series. Claesson (5) has reported the use of displacement analysis in the group separation of unsaturated, saturated and branched fatty acids using heptane as solvent and silica as adsorbent. Because of its obvious advantage, it was decided to again try displacement analysis of fatty acids in the saturated series, attempts at which had been unsuccessful in this laboratory (3) before the development of coupled filters.

EXPERIMENTAL

The interferometric adsorption analysis apparatus developed in this laboratory (6) was used in all experiments reported here. Coupled filters (7) offer the advantage of increasing the capacity of the filter and at the same time increasing the sharpness of the fronts, making the separation of components discernible. The mixer and improved coupled filters developed by one of us (8, 9) for use with the interferometric instrument were used in all cases. The mixer prevents the layering of the solution as it

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enters the cuvette and the consequent division and disappearance of the interference fringes when the front enters the cuvette.

All solutions were prepared in absolute alcohol. Filters were packed wet (7), and after thoroughly washing the filter column with absolute alcohol, the samples in solution were pressed into the filter and the volume of extruded alcohol was taken to be equal to the volume of impressed solution. The displacer solution was then forced through the filter at a rate of between 15 and 25 ml./hr.

RESULTS AND DISCUSSION

To use displacement analysis in separating fatty acids it was necessary to find a system in which the fatty acids could displace their next lower homologs and to find a suitable agent for displacing the more strongly adsorbed members of the series.

Cetyl pyridinium chloride, which has been successfully used as displacer for amino acids in this laboratory (10), was tried as displacer for fatty acids in alcohol solution. It was found that this substance was a strong displacing agent, at least partially displacing the normal saturated acids from 12 to 22 carbon atoms in length. Using 0.5% cetyl pyridinium chloride as displacer, a series of experiments was made in which a single fatty acid was displaced from Carboraffin Supra charcoal. The refractive indices of the displaced fatty acids in their stationary concentrations, or "characteristic heights," are given in Table I.

TABLE I

Characteristic Stationary Concentrations of Displaced Acids
(Carboraffin Supra charcoal, 0.5% cetyl pyridinium chloride)

Acid	Refractive index ($\Delta\mu \cdot 10^6$)
Dodecanoic (Lauric)	5.4
Tetradecanoic (Myristic)	9.9
Pentadecanoic	17.
Hexadecanoic (Palmitic)	25.
Heptadecanoic	32.
Octadecanoic (Stearic)	43.
Eicosanoic	58.
Docosanoic (Behenic)	100.

It is seen that the characteristic concentrations or "heights" are sufficiently different so that it should be possible to detect and separate members of this series in mixture because displacement of each acid by its higher homolog should be attainable.

Our first successful demonstration of displacement of fatty acids by their higher homologs was made using a coupled filter of 11.0 cc. total volume packed with Carboraffin Supra charcoal and Hyflo Super Cel (10) in a ratio of 1:1. Twenty mg. of myristic acid and 13 mg. of lauric acid in 4.0 ml. alcohol was pressed into the filter. A 0.5% solution of

palmitic acid was used as displacer. It is clear from the curve shown in Fig. 1 that palmitic acid displaced myristic acid, which, in turn, displaced the lauric acid. With this indication that fatty acids can at least partially displace their lower homologs, it was worth while to investigate other mixtures and other displacing agents.

A displacement of stearic and palmitic acids by cetyl pyridinium chloride was made, but when more complex mixtures were used, neces-
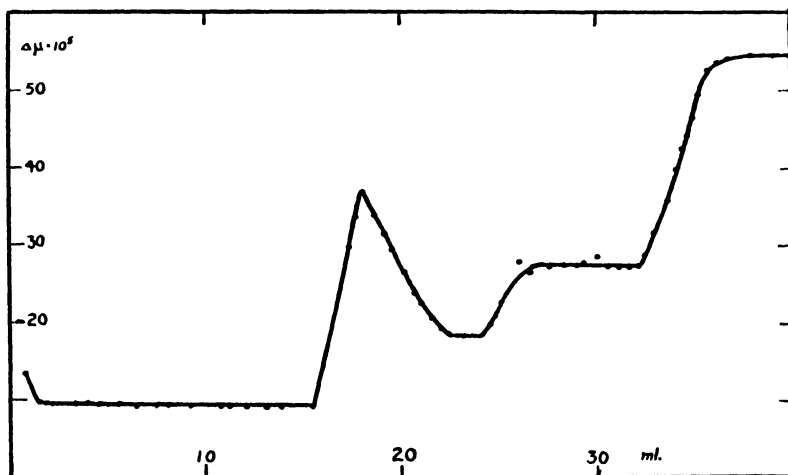
FIG. 1. Displacement of 20 mg. myristic acid and 13 mg. lauric acid in 4 ml. alcohol by 0.5% palmitic acid. Filter volume = 11,000 mm³. Carboraffin Supra + Hyflo Super Cel, 1/1.

sitating longer filters, difficulties were encountered. In Fig. 2 is shown a series of related experiments made on the same filter column which had been washed free of cetyl pyridinium chloride between experiments. The coupled filter, having a total volume of 10.6 cc., was packed with Carboraffin Supra charcoal and Hyflo Super Cel in a ratio of 1:1. The displacer was 0.5% cetyl pyridinium chloride. Curve 1 represents an experiment in which the sample contained 29 mg. stearic acid, 14 mg. palmitic acid, 7 mg. myristic acid, and 2.7 mg. lauric acid in 3.0 ml. displacer solution. The curve has 5 steps, of which at least one must be an impurity. To get a clue as to the identity of the various steps, the experiment was repeated with the omission of lauric acid. Sample 2 contained 36 mg. stearic acid, 17 mg. palmitic acid, and 9.1 mg. myris-
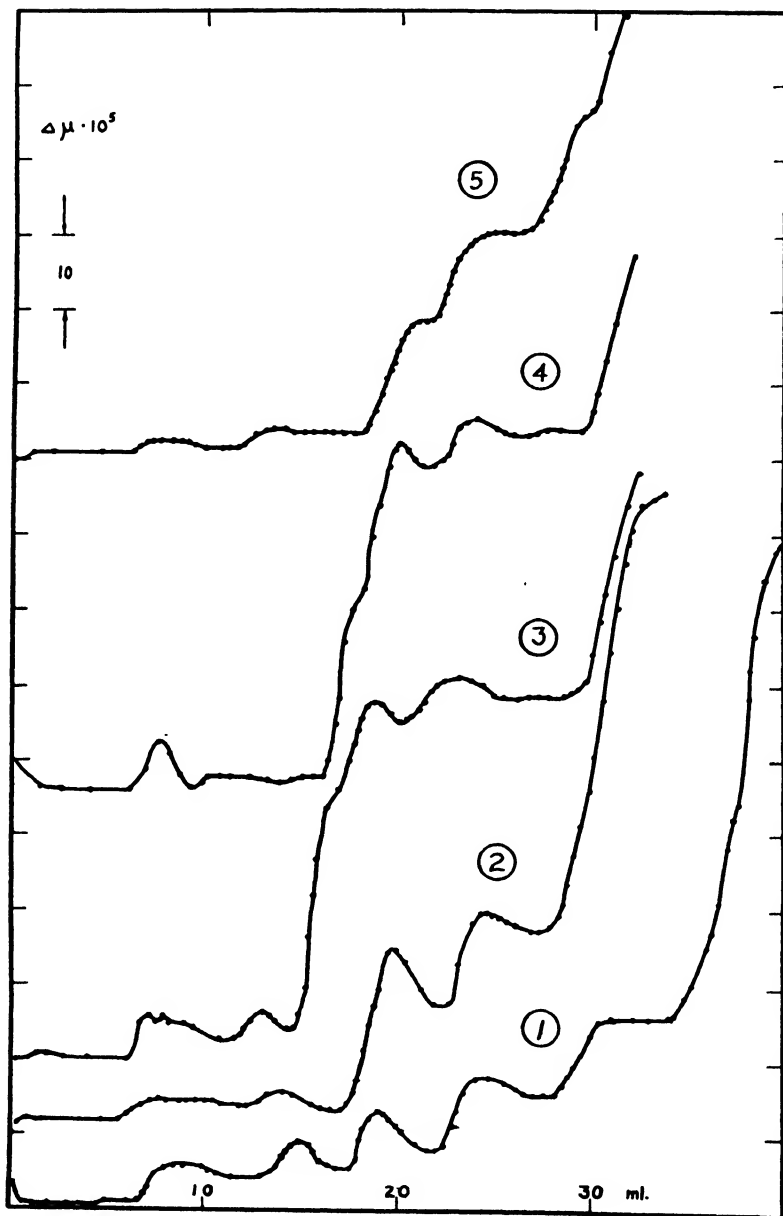
Fig. 2. A series of displacement experiments using the same filter column. Filter volume = 10,600 mm³. Adsorbent—Carboraffin Supra + Cel, 1/1. Displacer—0.5%

tic acid in 3.8 ml. displacer solution. It is clear that Curve 2 lacks the third step of Curve 1, which must then represent lauric acid. It was, therefore, concluded that the first two steps represent impurities in fatty acid samples used (Schering). However, the step representing stearic acid was missing, probably because of losses remaining on the charcoal. To bring forth this step, the experiment was repeated with a sample containing 74 mg. stearic acid, 17 mg. palmitic acid, and 9.1 mg. myristic acid in 3.8 ml. displacer solution. Curve 3 shows that with this large quantity of stearic acid its step became prominent, but the acids could not establish themselves in stationary concentration on so small a filter. The same effect was produced when eicosanoic acid was added in place of additional stearic acid. A sample containing 45 mg. eicosanoic acid, 45 mg. stearic acid, 15 mg. palmitic acid, and 6 mg. myristic acid in 3.0 ml. displacer solution is shown in Curve 4. Finally in Curve 5 we have a sample containing 45 mg. stearic acid, 15 mg. palmitic acid, and 6 mg. myristic acid in 3.0 ml. displacer solution. From this series of experiments it was concluded that displacement of the saturated acids is possible with cetyl pyridinium chloride, but that the losses sustained by the last present member of the series are particularly large, and that, in the case of eicosanoic acid, this loss must exceed 45 mg. on this filter.

This leaves much to be desired in the way of an analytical method. It is clear that the losses in the last component must be reduced. To that end, a search for a more suitable displacer was made. It was thought that a substance bearing the same charge as fatty acids would be more suitable than the cetyl pyridinium chloride. Sodium oleyl sulfate was tried but abandoned because inspection of the displacement diagrams showed that the recovery of stearic acid was even less than when cetyl pyridinium chloride was used.

Claesson concluded from frontal analysis experiments that picric acid and myristic acid were adsorbed on charcoal independently of each other (3, 11), and he recorded a very large retention volume for picric acid, indicating that it was very strongly adsorbed. Because of this property, and the fact that it bears the same charge as fatty acids,

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cetyl pyridinium chloride. (1) 29 mg. stearic acid, 14 mg. palmitic acid, 7 mg. myristic acid, and 2.7 mg. lauric acid in 3.0 ml. displacer. (2) 36 mg. stearic acid, 17 mg. palmitic acid, and 7 mg. myristic acid in 3.8 ml. displacer. (3) 74 mg. stearic acid, 17 mg. palmitic acid, and 9.1 mg. myristic acid in 3.8 ml. displacer. (4) 45 mg. eicosanoic acid, 45 mg. stearic acid, 15 mg. palmitic acid, and 6 mg. myristic acid in 3.0 ml. displacer. (5) 45 mg. stearic acid, 15 mg. palmitic acid, and 6 mg. myristic acid in 3.0 ml. displacer.

it was thought worth while to again attempt displacement of fatty acids with picric acid. It was first found that stearic acid is more nearly quantitatively displaced by picric acid than by cetyl pyridinium chloride, as judged from the respective displacement diagrams. In subsequent experiments it was found to be a very suitable displacer for mixtures as well as single components, and the use of cetyl pyridinium chloride was abandoned.
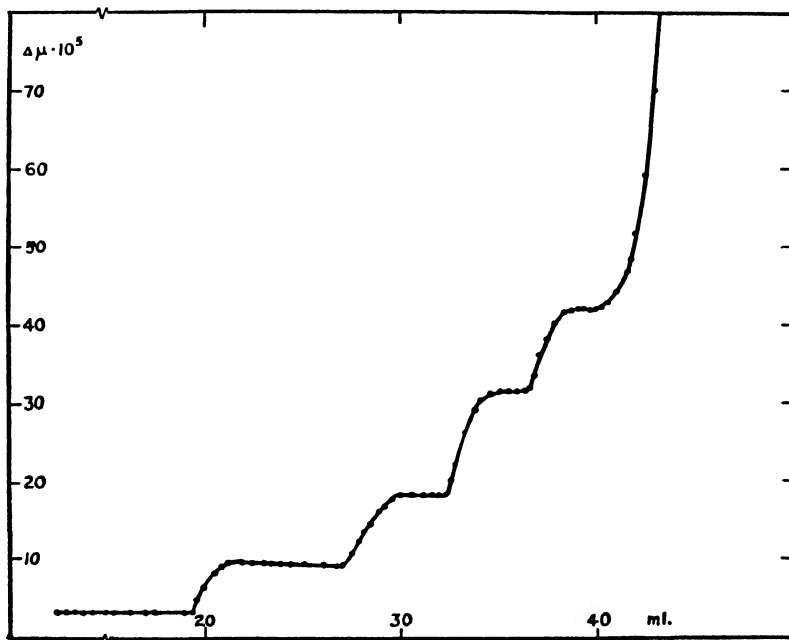
FIG. 3. Displacement of fatty acids by picric acid. Filter—11,400 mm³. Adsorbent —Carbo Activ + Hyflo Super Cel, 1/1. Displacer—0.5% picric acid. Sample—24 mg. stearic acid, 14.4 mg. palmitic acid, 8.4 mg. myristic acid, and 4.8 mg. lauric acid in 6.0 ml.

Picric acid can displace and separate mixtures of at least 4 components. One such experiment approaching an ideal displacement is shown in Fig. 3. The coupled filter of total volume 11.4 cc. was filled with Carbo Activ charcoal and Hyflo Super Cel (1/1). The sample contained 24 mg. stearic acid, 14.4 mg. palmitic acid, 8.4 mg. myristic acid, and 4.8 mg. lauric acid in 6.0 ml. absolute alcohol. The column was developed with 0.5% picric acid. From this and other similar experi-

ments it is clear that picric acid is a suitable displacing agent for lauric through stearic acids under these conditions. It appears, from our experience, that with suitable conditions of concentration, filter size, and suitable charcoal, the lower limit of usefulness can be extended. In an experiment using palmitic acid as displacer, a separation of octanoic, decanoic, and lauric acids was demonstrated. The upper limit of usefulness seems to be determined by the solubilities of the fatty acids. This limit can probably be extended by the use of the more soluble esters (3). It thus seems ultimately possible to use displacement as a means of separation and analysis of the more important saturated fatty acids in natural fats. However, it should be emphasized that much investigation is necessary before this goal is reached.

The use of picric acid as displacer afforded the opportunity of investigating the sharpness of division of two components in a displacement analysis. Experiments were made in which the solution emerging from the apparatus was fractionated into numerous small samples. The light absorption of picric acid was measured and an independent measure of the increase in picric acid was thus obtained. It was found that the front, as observed interferometrically, appeared at the same time as that observed spectrophotometrically.

To gain some information regarding the sharpness of the rear of a component, a conjugated unsaturated fatty acid was used. The isomer of 10,12-linoleic acid, melting at 8°C., was used as a component and its light absorption at 2340 Å was used as an independent measure of its concentration. It was found again that the front was sharp, appearing at the point indicated by an increase in index of refraction. However, the rear exhibited a tail extending for a considerable distance into the displacers, which in these experiments were higher saturated acids. In Fig. 4 is shown an experiment in which conjugated linoleic acid is displaced by 0.1% behenic acid. The concentration of conjugated acid as measured spectrophotometrically and the refractive index measurements are plotted together. The scales have been adjusted so that the horizontal plateaus of both plots coincide. It is seen that the front observed interferometrically is that due to conjugated acid. It is also seen that the drop in conjugated linoleic acid upon the appearance of behenic acid is quite sharp, but that it does not reach zero during the course of the experiment. It thus appears that, in a series of components displacing each other, only the first component is pure.

The calculated recoveries of conjugated fatty acid under these conditions are only about 70%, and it appears that, with this charcoal at least, a sizeable portion of the acid remains on the carbon. We wish to point out, therefore, that the separations observed interferometrically
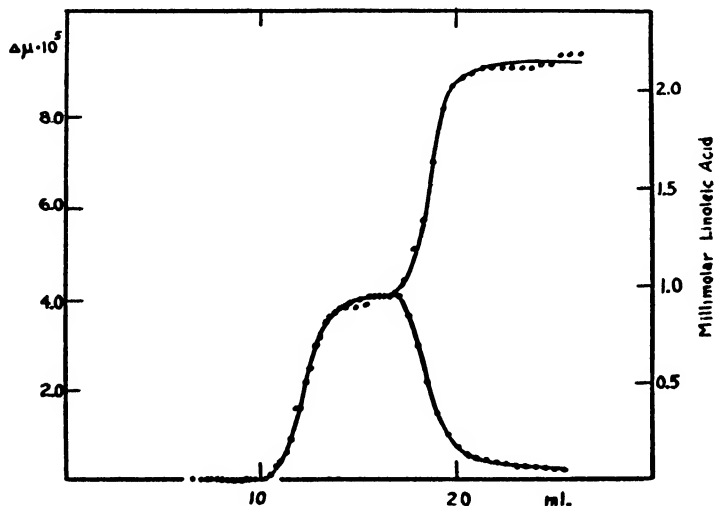
FIG. 4. Displacement followed spectrophotometrically. Filter—1340 mm³. Adsorbent—Carbo Activ + Hyflo Super Cel, 1/1. Displacer— 0.1% behenic acid. Sample—2.5 mg. 10,12-linoleic acid in 5 ml.

and discussed above, although real, are probably only partial. It should, therefore, be understood that the words separation and displacement, as used here, do not imply total separation into pure components, or quantitative displacement. The emphasis in this present study has been to obtain a separation of fatty acids by displacement as judged from interferometric data, and less attention has been centered to date on obtaining quantitative recoveries. It should also be pointed out that, in these experiments, it has been necessary to choose suitable quantities of fatty acids to obtain displacement diagrams in which the acids could establish themselves in stationary concentrations. In its present state of development, displacement analysis is not suitable for analysis of unknown samples. It will be necessary to find conditions under which small quantities of one acid can be detected in the presence of large quantities of others. To accomplish this it may be necessary to

adopt the use of low concentrations of displacer and longer filters, conditions which give curves with long, low steps which lend themselves to more accurate calculations than the rather tall narrow steps shown here.

At present, however, it is possible to use displacement analysis as an identifying tool for fatty acids, for it has been found that the "characteristic heights" of the fatty acids are the same, within experimental error, whether they be displaced singly or from mixtures. Within limits, the relative concentrations of fatty acids can also be determined. Displacement, as a preparative method, shows promise, but it should be remembered that, in this connection, components other than the first may be impure because of the tailing-off phenomenon shown above.

Displacement analysis shows promise as an analytical tool, but much fundamental work remains to be done before it may be applied generally to the analysis of fat. Further work is planned on the problems raised during this investigation.

SUMMARY

It has been demonstrated that saturated fatty acids can displace their lower homologs from charcoal, making the application of displacement possible in the fatty acid series.

Cetyl pyridinium chloride has been found to be a displacing agent for fatty acids in alcoholic solution, but with this displacer relatively large quantities of the higher acids are lost on the charcoal.

Picric acid is the best displacing agent so far used. Using this displacer, it has been demonstrated that stearic, palmitic, myristic and lauric acids can be separated by displacement, as judged from displacement diagrams.

An independent measure of the appearance of the fronts indicates that the increase in refractive index coincides with the appearance of a new substance in the cuvette. The rear of a component drops off rather sharply but does not reach zero. This indicates that the only pure component in a displacement experiment is the first component.

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Biochemical Mechanisms in the Respiration of the *Avena* Coleoptile[1]

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INTRODUCTION

The coleoptile of the oat seedling has become a classical object in the investigation and determination of the class of plant growth hormones known as the auxins. The growth of the oat coleoptile is regulated by the amount of auxin, for example, of indoleacetic acid, which is present in the growing regions of the organ and this amount may be altered by artifical application of auxin to the tissue. The growth response of the oat coleoptile to auxin is known to be intimately related to the respiration of the plant. Thus, when respiration is inhibited by HCN, or by absence of O_2, growth is proportionately reduced (Bonner, 1933). The nature of this relationship between growth and respiration has been viewed in different ways. Thus, it may be that in respiration a substance or condition essential for the functioning of auxin is produced (Bonner, 1936). On the other hand, Commoner and Thimann (1941) have suggested that the function of auxin in promoting growth may be involved directly in a respiratory process. In either case, a closer analysis of the role of auxin in growth of the *Avena* coleoptile requires an understanding of the biochemical mechanisms involved in the respiration of this organ. The present paper is concerned with a survey of the respiratory processes of the *Avena* coleoptile.

MATERIALS AND METHODS

Etiolated oat seedlings for all of the experiments reported here were grown in a temperature-controlled dark room at 26°C. Orange light was used as needed for manipulation of the plants. Seeds (unhusked) of *Avena sativa*, variety *Siegeshafer,*

were planted in washed quartz sand contained in flats. After 4 days the coleoptiles were approximately 2 cm. tall and the plants were ready for use. The coleoptiles were cut from the mesocotyls, 2–3 mm. apical tips removed, and the primary leaf removed to leave the hollow coleoptile cylinder. This tissue was used in various ways as described below. Measurements of gas exchange were made in a Warburg respirometer using conical vessels of approximately 16 cc. volume and a bath temperature at 30°C. All substrates were supplied in 0.05 M KH$_2$PO$_4$, pH 4.5, unless otherwise noted. Penetration of substrate was not promoted by infiltration as was found for spinach leaves (Wildman and Bonner, 1946) and this operation was therefore omitted. Enzyme preparations were made in all cases by grinding the tissue in a mortar with an equal weight of buffer, in general 0.1 molal phosphate buffer, pH 6.5. All manipulations incident to the preparation of the active extracts were carried out at 0°C.

Preliminary Experiments

Excised sections of *Avena* coleoptiles continue to respire over a considerable period of time when they are suspended in phosphate buffer and placed in Warburg vessels (Bonner, 1936). The respiratory quotient of such tissue is approximately 1 (Bonner, 1936), and it may be presumed that carbohydrate is the normal endogenous substrate. Table I shows that, in sections freshly cut from the coleoptiles of seedling plants, the

TABLE I

Influence of Sucrose on the Respiratory Rate of Avena Coleoptile Sections
Which Have Been Subjected to Various Pretreatments

Measurements based on gas exchange during 2nd hour

Expt. No.	Pretreatment	Rate of respiration. mm.^{3} O$_2$/200 mg. fresh wt./hr.		Per cent increase
		No sucrose	1% sucrose	
349	None	76.0	93.0	22
362	Endosperm removed 48 hours before	20.4	36.0	77
356	Endosperm removed 96 hours before	21.2	54.5	157
359	Sections soaked 24 hours in water	50.0	70.0	40

addition of further carbohydrate in the form of sucrose has only a small effect on respiratory rate, so that, under normal conditions, substrate does not appear to be a limiting factor in respiration. For this experiment 20 sections, each 7.5 mm. long, were suspended in 0.05 M phosphate buffer of pH 4.5 in each Warburg vessel. On the other hand, if coleoptiles were depleted of their normal endogenous respiratory sub-

strate, considerable increases in respiratory rate are achieved by the addition of sucrose. Depletion was attained by removal of the endosperm from germinated oat seeds. After the seedlings had grown a further 48 hours, sections were removed from the coleoptiles and subjected to measurements of respiratory rate as before. Table I shows that whereas, in normal nondepleted coleoptiles, respiration was increased by only approximately 20% by addition of 1% sucrose, in the coleoptiles from de-endo-spermed plants addition of sucrose increased respiration by as much as 2.7 times.

Coleoptiles were also depleted by allowing excised sections from normal plants to soak for 24 hours in water at 26°C. before the respiratory measurements were carried out. With such sections respiration was increased over that of the control plants not supplied with sucrose by 40%. It may be concluded on the basis of the experiments of Table I, which have been repeated and confirmed, that *Avena* coleoptile sections can utilize sucrose as a substrate for respiration.

Initial Breakdown of Carbohydrate

It has been shown in earlier work that the initial breakdown of carbohydrate proceeds through pyruvic acid in barley leaves (James and James, 1940), in spinach leaves (Bonner and Wildman, 1946), and possibly in other plant tissues as well. That breakdown of carbohydrate to pyruvic acid may also be a normal step in the respiration of the *Avena* coleoptile is indicated by the fact that depleted coleoptile sections are able to respire at the expense of added pyruvate as is shown in Table II. For the experiments of Table II, coleoptiles were depleted of endogenous substrate as before and were then suspended in 0.05 *M* phosphate buffer containing 5 mg./cc. of pyruvate, a concentration previously determined to be optimal. Table II shows that the depleted coleoptiles show an increase in respiratory rate of 57–94% in the presence of pyruvate. Pyruvate would appear to be at least as suitable a substrate for respiration as sucrose.

The respiration of the *Avena* coleoptile, unlike that of the spinach leaf (Bonner and Wildman, 1946) and other tissues, is but little inhibited by fluoride in low concentrations as is shown in Table III. Fluoride concentrations of 0.1 mg. NaF/cc. give only 36% inhibition of the respiration of *Avena* coleoptiles, whereas the respiration of spinach leaves was found to be approximately 80% inhibited by the same concentration. Fluoride is known to interfere with the respiration of many tissues by inhibition of the enzyme enolase which is responsible for one of the steps in the conversion of hexose to pyruvate, *e.g.*, the conversion of 2-phosphoglycerate to phosphopyruvate. That fluoride inhibition, such as it is, may occur at a similar level in the *Avena* coleoptile is indicated by the experiment of Table IV. In this experiment sections were

TABLE II

Influence of Pyruvate in Increasing the Respiratory Rate of Excised
Sections of Avena Coleoptiles

Measurements based on gas exchange during 2nd hour

Expt. No.	Pretreatment	Gas exchange in mm.³ O₂/200 mg. fresh wt./hr.		Per cent increase
		No pyruvate	Pyruvate 5 mg./cc.	
362	Endosperm removed 48 hours before	20.4	36.0	77
354	Endosperm removed 72 hours before	17.9	34.7	94
369	Sections soaked 24 hours in H₂O	53.8	84.4	57

placed either in solutions containing NaF, 0.1 mg./cc., or NaF to which pyruvate had been added. The addition of pyruvate (at a concentration of 2.5 or 5.0 mg./cc., previously determined to be optimal) increased rate of oxygen uptake in the presence of fluoride. Sucrose on the other hand did not increase respiration in the presence of fluoride. In *Avena*, as in spinach leaves, fluoride inhibition of respiration would, therefore, appear to take place at the level between sucrose and pyruvate.

TABLE III

Inhibition of the Respiration of Avena Coleoptile Sections by
Various Concentrations of NaF

Expt. 270. Plants suspended in 0.05 M phosphate buffer pH 4.5.
Measurements during 2nd hour

Conc. of NaF. mg./cc.	0	0.01	0.03	0.1	0.3	1.0
Conc. of NaF. M.	0	2.4×10^{-4}	7.2×10^{-4}	2.4×10^{-3}	7.2×10^{-3}	2.4×10^{-2}
Gas exchange. mm.³ O₂/ 200 mg. fresh wt./hr.	61.4	59.0	50.0	39.0	28.4	15.8
Per cent inhibition	—	4	19	36	54	74

The *Avena* coleoptile contains the phosphorylated compounds typical of phosphorylative glycolysis in yeast and in animal tissues. Table V gives data on the concentrations of fructose diphosphate, fructose-6-phosphate, and glucose-1-phosphate found in plants of the age used in these experiments. These determinations were made

TABLE IV

Partial Reversal of Fluoride Inhibition of Respiration of Avena Coleoptile Sections by Pyruvate

Expt. 268. Measurements during 2nd hour

Vessel	Conc. of NaF	Conc. of substrate	Oxygen uptake mm.^{3}O$_2$/200 mg./hr.
1	None	None	61.5
2	0.1 mg./cc.	None	42.5
3	0.1 mg./cc.	Pyruvate 2.5 mg./cc.	55.6
4	0.1 mg./cc.	Sucrose 10 mg./cc.	44.7

according to the methods of Umbreit *et al.* (1944) and, while they may not be fully quantitative, they do indicate qualitatively the presence in *Avena* of these intermediates in phosphorylative sugar breakdown. Albaum and Umbreit (1943) have also reported the presence of phosphorylated sugars and sugar derivatives in whole *Avena* seedlings.

TABLE V

Amounts of Various Phosophorylated Compounds Found in Avena Coleoptile Tissue

Expt. 247

Compound	Mg./g. fresh wt.
Fructose-1,6-diphosphate	0.77
Fructose-6-phosphate	0.48
Glucose-6-phosphate	0.00
Glucose-1-phosphate	0.15
Inorganic phosphate	0.15

Indication that enzymes essential for phosphorylative carbohydrate breakdown are present in *Avena* is given by the experiment of Table VI, which shows that cell-free juice of *Avena* coleoptiles is able to phosphorylate glucose with the production of fructose diphosphate.

For this experiment 50 g. of *Avena* coleoptiles were ground in a blendor with 50 cc. of phosphate buffer, pH 6.5. The whole brei was centrifuged and the resultant juice used as the enzyme. To this enzyme solution was added NaF, 1 mg./cc., to inhibit breakdown of fructose diphosphate by the phosphatase of the extract. In separate reaction mixtures glucose, and glucose plus ATP, were further added as reactants. Aliquots were removed after 0, 1, and 2 hours and the amounts of fructose diphosphate determined by the methods of Umbreit *et al.* Table VI shows that the *Avena*

TABLE VI

Formation of Fructose Diphosphate by Avena Coleoptile Enzyme Extracts

All mixtures contained NaF in conc. of 1 mg./cc., 0.05 M phosphate buffer
pH 6.5 and enzyme from 375 mg. tissue/cc. Expt. 262

Reaction mixture	Mg. FDP/1 g. original tissue		
	0 hours	1 hour	2 hours
No substrate added	0.77	0.67	0.81
Glucose alone (10 mg./cc.)	—	1.16	1.03
Glucose + ATP (2.5 mg./cc.)	—	1.33	1.65

extract converted an appreciable although small portion of the added glucose to fructose diphosphate, the amount of FDP in the extract being increased by 50% over a 1 hour period. Additions of ATP gave a further increase in FDP accumulation, amounting to as much as 114% over the intial amount of FDP.

Three lines of evidence suggest that in *Avena* the initial breakdown of carbohydrate in respiration may follow a phosphorylytic pathway through fructose diphosphate and ultimately to pyruvate. In the first place, the phosphorylated intermediates are normally found in the respiring *Avena* coleoptile. In the second place, enzyme preparations from *Avena* are capable of forming a key intermediate, *e.g.*, fructose diphosphate, from glucose, and formation of this intermediate is increased in the presence of ATP. And thirdly, although the respiration of *Avena* is moderately insensitive to inhibition by fluoride, still such inhibition as does occur can be largely overcome by further addition of pyruvate and cannot be overcome by addition of sucrose, indicating that fluoride in *Avena*, as in other organisms, may inhibit a step in the phosphorylative breakdown of hexose to pyruvate.

The Terminal Oxidase

The enzyme which carries out the final uptake of oxygen in plant respiration is now known to differ in different plant tissues. In the potato tuber polyphenol oxidase performs this function (Boswell and Whiting, 1938; Boswell, 1945; Baker and Nelson, 1943) and the same is true of the sweet potato (Walter and Nelson, 1945) and of the spinach leaf (Bonner and Wildman, 1946). In other tissues, however,

a cytochrome-cytochrome oxidase system is operative as has been shown for pollen (Okunuki, 1939), and, most elegantly, by Goddard for the wheat embryo (1944). In the *Avena* coleoptile also, a cytochrome-cytochrome oxidase system is operative as will be shown below. *Avena* coleoptile extracts are devoid of an active polyphenol oxidase as is shown by the inability of such extracts to oxidize catechol or dihydroxyphenylalanine at an appreciable rate. In addition, the rate of oxygen uptake of *Avena* coleoptiles is not influenced by addition of catechol or of dihydroxyphenylalanine as is the case with potato tuber (Boswell and Whiting, 1938) and with spinach leaves (Bonner and Wildman, 1945). On the other hand *Avena* coleoptile extracts rapidly oxidize cytochrome c as is shown in the data of Fig. 1.

TIME: MINUTES
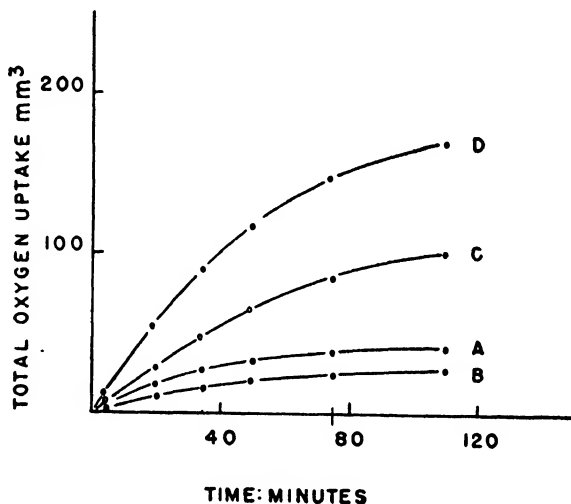
Fig. 1. Oxygen uptake of whole *Avena* cytoplasm in presence of added cytochrome c and/or p-phenylenediamine. Each reaction mixture contains enzyme preparation from 1.5 g. tissue in 2.5 cc. 0.1 M phosphate buffer, pH 6.5. A—enzyme alone, B—enzyme + cytochrome c 1.1×10^{-7} mols/cc., C—enzyme + p-phenylenediamine 0.1 mg./cc., D—enzyme + cytochrome c + p-phenylenediamine. Expt. 386.

Cytochrome was prepared by the method of Umbreit *et al.* from beef heart, and the solution used contained 0.28×10^{-7} mols/ml. *Avena* coleoptile brei was prepared by grinding *Avena* coleoptiles as described above, centrifuging the brei, and the whole supernatant used as the enzyme preparation. This preparation has a small initial endogenous oxygen uptake. When cytochrome is added, the rate of oxygen uptake is somewhat depressed. If p-phenylenediamine is added as a substrate the rate of oxygen

uptake is increased, and if cytochrome is further added, oxygen uptake is increased by a factor of 2 over that found with p-phenylenediamine alone. The extract is, therefore, able to oxidize p-phenylenediamine, a reagent known to be oxidized by cytochrome c in the presence of cytochrome oxidase. The reaction is limited by the amount of cytochrome c present in the extract, since, when further cytochrome c is added, the reaction is greatly accelerated. The presence of cytochrome oxidase in whole oat seedlings has previously been demonstrated by Albaum and Eichel (1943).

The respiration of *Avena* coleoptile sections is known from earlier work (Bonner, 1936) to be inhibited by cyanide, 10^{-4} M cyanide giving approximately 50% inhibition of oxygen uptake. This behavior, while not specific for respiration involving cytochrome oxidase, does indicate the participation of a heavy metal protein. This same conclusion may also be drawn from the fact that the respiration of whole oat seedlings is inhibited by sodium azide (Albaum and Eichel, 1943).

The Oxidation of Pyruvate

It has been shown in an earlier paper (Bonner and Wildman, 1946) that, in the spinach leaf, the oxidation of pyruvate in respiration may proceed through a series of reactions involving the plant acids and resembling, in general, the Krebs cycle. The behavior of *Avena* coleoptiles shows certain differences from that of the spinach leaf. Nevertheless, respiration in this tissue also would appear to involve the metabolism of organic acids as is indicated by several lines of investigation. In the first place, although sections of normal *Avena* coleoptiles respond only slightly to added organic acids with increased respiration, coleoptiles which have been depleted by soaking in water for 24 hours show moderately large increases in rate of oxygen uptake in the presence of organic acids, as is shown in Table VII. Fumarate, malate, succinate, isocitrate, and α-ketoglutarate, all acids which participate in the Krebs cycle, are capable of increasing rate of oxygen uptake in depleted *Avena* coleoptile sections. The work of Berger and Avery (1943) has shown further that typical dehydrogenases for the oxidation of malate and isocitrate are present in the *Avena* coleoptile. These dehydrogenases require cozymase or coenzyme II as do the dehydrogenases of other tissues. The observations were confirmed in the present work. Berger and Avery have shown also that the dehydrogenases of *Avena*, and alcohol dehydrogenase in particular, are sensitive to inhibition by iodoacetate. Fig. 2 shows that the respiration of *Avena* coleoptile sections is likewise strongly inhibited by iodoacetate, an

TABLE VII

*Effect of Various Organic Acids on the Rate of Oxygen Uptake of
Avena Coleoptile Sections*

Measurements during 2nd hour. All sections depleted by
soaking 24 hours in H_2O

Expt. no.	Substrate added	O_2 uptake in mm.³ O_2/200 mg./hr.	Per cent increase
424	None	48.0	—
	Succinate 5 mg./cc.	70.4	46
372	None	48.0	—
	Succinate 5 mg./cc.	68.0	42
	Fumarate 5 mg./cc.	60.0	25
415	None	35.4	—
	α-Ketoglutarate 2.5 mg./cc.	45.2	28
413	None	39.0	—
	Isocitrate 2.5 mg./cc.	48.0	23
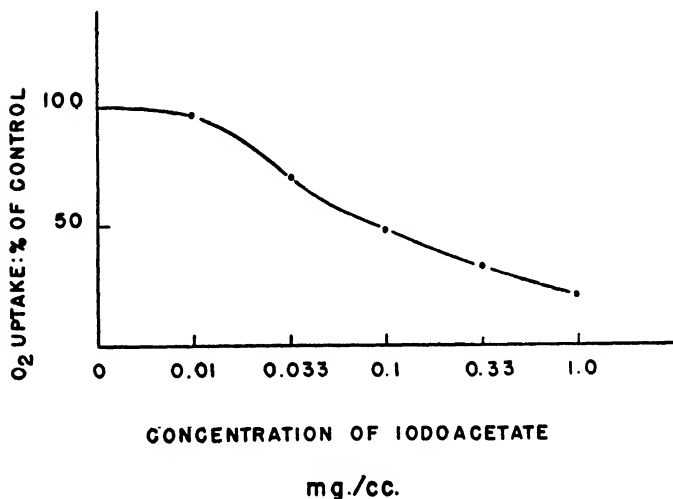
Fig. 2. Inhibition of respiration of *Avena* coleoptile sections by iodoacetate.
Figures represent values during 2nd hour in solutions of concentrations stated.
Expt. 352.

observation confirming that of Commoner and Thimann (1941). The data of Table VIII show that malonate, an inhibitor of succinic dehydrogenase, exerts an inhibiting effect on the oxygen uptake of *Avena* coleoptiles although only in moderately high concentration. Thus, a concentration of malonate of 5 mg./cc., which inhibits the respiration of spinach leaves by 90% or more, inhibited the respiration of *Avena* coleoptile sections by only 12%. This slight inhibition of O_2 uptake by malonate is related to the nutritional status of the coleoptile, since in starved sections the same concentration of malonate may give over

TABLE VIII

Effect of Malonate on the Rate of Oxygen Uptake by Avena Coleoptile Sections
Measurements during 2nd hour

Expt. no.	Pretreatment	Malonate conc.		O_2 uptake in mm.^{3} O_2/200 mg./hour	Per cent inhibition
		mg./cc.	Molal		
265	None	0.0	0.0	69.6	—
		5.0	5×10^{-2}	61.2	12
		10.0	10×10^{-2}	27.4	61
		20.0	20×10^{-2}	18.9	73
369	Soaked 24 hours in H_2O	0.0	0.0	56.2	—
		5.0	5×10^{-2}	20.0	64
370	Soaked 24 hours in H_2O	0.0	0.0	52.0	—
		5.0	5×10^{-2}	26.0	50
364	Endosperm removed 48 hours before	0.0	0.0	17.9	—
		5.0	5×10^{-2}	1.3	93

90% inhibition. Degree of malonate inhibition also varied with age and status of the coleoptile. Malonate inhibition was found to be dependent on the pH of the solution in which the malonate was supplied, a result similar to that reported for the spinach leaf (Wildman and Bonner, 1946). From the fact that malonate inhibition as a function of pH follows closely the titration curve of malonate, it was earlier concluded that this effect is owing to penetration only of the monovalent malonate ion into the cell. Thus, malonate was strongly inhibitory at pH 4.5, only slightly inhibitory at pH 5.5, and ineffective at pH 6.5. The ineffectiveness of malonate as a respiratory inhibitor in *Avena*, pre-

viously reported by Albaum and Eichel (1943), is no doubt owing to the fact that they worked at pH 6.0. The fact that the respiration of *Avena* coleoptiles is inhibited by malonate is a strong indication that succinic dehydrogenase participates in the respiration of this tissue.

Malonate inhibition of succinic dehydrogenase is, in general, a competitive inhibition, and is, owing to the similarity between malonate and succinate, the normal substrate for the enzyme. Malonate inhibition of respiration, if due to inhibition of succinic dehydrogenase, should therefore be reversed to some extent, at least by the further addition of succinate. The small amount of inhibition of respiration brought about in nondepleted *Avena* coleoptiles by the addition of low concentrations of malonate does not appear, however, to be offset by addition of succinate, perhaps owing to a high concentration of succinate and succinate precursors already in the tissue (see above). In depleted sections however, malonate inhibition is readily offset by added succinate as shown in Table IX. In the experiment of Table IX depleted sections were supplied with pyruvate which is utilized as shown in Table II. The oxidation of pyruvate is inhibited by malonate. The respiration of sections supplied with malonate, pyruvate, and succinate is almost twice as large as respiration in the presence of malonate and pyruvate alone. The data of Table IX are, then, in accord with the view that pyruvate oxidation proceeds through a system involving succinic dehydrogenase. The role of succinate in pyruvate oxidation can be fulfilled also by fumarate, isocitrate, and α-ketoglutarate as shown in Table IX. Since the reversal of malonate inhibition is a property specific to succinate, it must be concluded that these acids are oxidatively converted to succinate.

Further evidence that malonate acts by blocking succinic dehydrogenase is given by the experiment of Table X which shows that, in coleoptiles treated with malonate, accumulation of succinate occurs.

For this experiment *Avena* coleoptile sections were allowed to float for 8 hours in solutions containing either phosphate buffer (pH 4.5) alone or buffer plus malonate, 5 mg./cc. At the end of the experimental period the sections were rapidly dried at 70°C., ground, acidified, and extracted with ether. After removal of the ether, the whole acid extract was oxidized with alkaline permanganate for the removal of plant acids other than succinate and the residual succinate determined manometrically using sheep heart succinoxidase. Table X shows that tissue floated in buffer alone contained a small amount of succinate, 0.80 mg./g. on a dry weight basis. In tissue treated with malonate the level of succinate was increased to approximately 3 times

TABLE IX

Effect of Various Organic Acids on the Inhibition of Pyruvate Metabolism by Avena Coleoptile Sections by Malonate

All sections depleted by soaking in water for 24 hours

Expt. no.	Addenda to sections		Malonate	O₂ uptake mm.³/ 200 mg./hr.
	Pyruvate	Plant acid		
366	5 mg./cc.	None	None	45.0
	5 mg./cc.	None	5 mg./cc.	19.2
	5 mg./cc.	Succinate 2.5 mg./cc.	5 mg./cc.	33.4
408	2.5 mg./cc.	None	None	54.2
	2.5 mg./cc.	None	5 mg./cc.	18.0
	2.5 mg./cc.	Fumarate 2.5 mg./cc.	5 mg./cc.	48.2
410	2.5 mg./cc.	None	None	56.6
	2.5 mg./cc.	None	5 mg./cc.	22.0
	2.5 mg./cc.	α-Keto-glutarate 2.5 mg./cc.	5 mg./cc.	29.6
413	2.5 mg./cc.	None	None	48.0
	2.5 mg./cc.	None	5 mg./cc.	12.0
	2.5 mg./cc.	Isocitrate 2.5 mg./cc.	5 mg./cc.	21.0

this level. Tissue floated on buffer containing fumarate, on the contrary, contained no more succinate than untreated tissue.

In animal tissue, Krebs and Johnson have shown that succinate is produced oxidatively from fumarate and other of the acids of the Krebs cycle, and that, in the presence of malonate, the supplying of fumarate leads to increased accumulation of succinate. This is also true in the case of the depleted *Avena* coleoptile as shown in Table X.

For this experiment *Avena* coleoptile sections were first depleted by being allowed to soak for 24 hours in water. They were then transferred to phosphate buffer, pH 4.5, containing either no addition, malonate, or malonate plus fumarate. After an incubation period of 7 hours the sections were dried, the acids extracted, oxidized with permanganate, and succinate determined as before. The succinate content of the depleted control tissue was essentially similar to that of the nondepleted tissue, in-

dicating that depletion involves depletion of substrates other than succinate. In the presence of malonate an accumulation of succinate occurred, although to a considerably lower value than was the case with nondepleted malonate-treated tissue. In the presence of both malonate and fumarate, however, succinate was accumulated to a level almost 7 times that of the control tissue and 4 times that of the tissue treated with malonate alone.

TABLE X

Accumulation of Succinate in Avena Coleoptile Sections Poisoned with Malonate

Incubation time 7–8 hours. All treatments given
in 0.05 M phosphate buffer, pH 4.5

Expt. no.	Treatment	Addenda to incubation solution	Succinic acid mg./g. dry tissue
379	Not depleted	None	0.80
		Malonate 5 mg./cc.	2.35
		Fumarate 1 mg./cc.	0.80
420	Depleted	None	0.93
		Malonate 5 mg./cc.	1.63
		Malonate + fumarate 2.5 mg./cc.	6.40
436	Depleted	None	1.05
		Malonate 5 mg./cc.	1.30
		Malonate + α-ketoglutarate 5 mg./cc.	4.96

The oxidative production of succinate from fumarate has been taken by Krebs (1934) as important evidence for the establishment of the concept of the participation of an organic acid cycle in respiration. Addition of fumarate to nondepleted coleoptiles did not result in increased succinate accumulation, either in the presence or in the absence of malonate. This may again be related to the fact indicated in Tables VII and VIII that *Avena* coleoptiles normally contain a large, or at least a nonlimiting supply, of organic acid metabolites. Additional evidence that *Avena* coleoptiles are normally not limited in their metabolism by available organic acid is the fact that malonate inhibition of respiration is not normally reversed by added fumarate, malate, citrate, or isocitrate. Apparently, the amounts of these acids already in the tissue are sufficient to saturate the several dehydrogenase systems. In depleted tissues, on the other hand, as shown in Table IX, not only fumarate but also other acids of the Krebs cycle do offset

malonate inhibition and are hence presumably also converted to succinate. Further evidence on this point is given by the last experiment of. Table X. In this experiment, depleted *Avena* coleoptiles were supplied with either malonate or malonate plus α-ketoglutarate. At the expiration of 8 hours, the coleoptiles were dried and extracted with acid ether as before. Succinic acid was determined as before on permanganate-oxidized aliquots of the ether extract. Since α-ketoglutarate is oxidized to succinate by this procedure, a parallel determination of α-ketoglutarate in the original ether extract was carried out by precipitating the keto acids with 2,4-dinitrophenylhydrazine. The acid ether extract of α-ketoglutarate-treated plants contained negligible amounts of α-ketoglutarate, although it did contain another as yet unidentified carbonyl-containing compound. The results given in Table X show that, in malonate-inhibited coleoptiles, accumulation of succinate can be brought about by α-ketoglutarate as by fumarate.

DISCUSSION

The evidence presented above permits of a generalized outline of the systems involved in the respiration of the *Avena* coleoptile. These systems appear to be generally similar to those found in other plant and animal tissues. The terminal oxidase, through which uptake of oxygen is achieved, is cytochrome oxidase. Cytochrome oxidase capable of oxidizing beef heart cytochrome c is found in the coleoptile. In this regard the coleoptile differs from the spinach leaf previously studied (Bonner and Wildman, 1947) in which the terminal oxidase is polyphenol oxidase and from which both cytochrome oxidase and cytochrome are absent. Cytochrome oxidase is, however, the terminal oxidase of the wheat embryo (Goddard, 1944) and of pollen (Okunuki, 1939). In the higher plants there is not the uniformity in terminal oxidase type which is characteristic of the tissues of higher animals.

The initial breakdown of hexose in the *Avena* coleoptile is phosphorylative as indicated by the fact that hexose can be phosphorylated at least as far as fructose diphosphate by cell-free coleoptile preparations and this phosphorylation is promoted by the presence of ATP. Additional evidence concerning the nature of hexose breakdown is the fact that hexose monophosphates and fructose diphosphate are normal constituents of the coleoptile. It has not been directly shown that pyruvate is actually the final product of the initial hexose break-

down but it has been shown that pyruvate can be respired by *Avena* coleoptiles which have been partially depleted of endogenous substrates. It will be of interest in future work to attempt to identify and characterize the individual enzymes which are involved in the transformation of hexose to pyruvate in the coleoptile.

The oxidation of pyruvate by the *Avena* coleoptile is inhibitable by malonate, an inhibitor of succinic dehydrogenase. This fact indicates at once that pyruvate is oxidized through the intermediary of succinate, a conclusion confirmed by direct analyses, which show that, in the presence of malonate, succinate accumulates in respiring tissues. Malonate inhibition of pyruvate oxidation can be partially offset by the addition of succinate, indicating the competitive nature of malonate inhibition in the *Avena* coleoptile. In addition, fumarate, isocitrate, and α-ketoglutarate, all acids of the Krebs cycle, possess the ability of offsetting to some degree malonate inhibition of pyruvate oxidation. This fact is in agreement with the hypothesis that the acids named are converted oxidatively to succinate, a hypothesis which is shown analytically to be correct in the case of fumarate and α-ketoglutarate. Enzymes for the interconversion of the organic acids are known from the *Avena* coleoptile in the cases of isocitric dehydrogenase, aconitase, fumarase, and malic dehydrogenase. Succinic dehydrogenase, while indirectly demonstrable *in vivo*, has, however, not as yet been obtained *in vitro* from coleoptile preparations, and this enzyme would appear to be exceedingly labile, as previously found in the case of the succinic dehydrogenase of spinach leaves (Bonner and Wildman 1946). The data taken together indicate that, in the *Avena* coleoptile, oxidation of pyruvate may proceed through a series of steps generally similar to the Krebs cycle.

No consideration has been given in this work to the role of flavoproteins in the respiration of the *Avena* coleoptile. Berger and Avery (1943, 1944) have shown, however, that the activity of dehydrogenase-substrate-thionin systems, prepared with enzymes from the coleoptile, is greatly enhanced by addition of milk xanthine oxidase, which reacts with reduced cozymase, or by addition of cytochrome reductase, which reacts with reduced triphosphopyridine nucleotide. The coleoptile contains riboflavin (Berger and Avery, 1943), and it is possible that this may be bound in a flavoprotein. Actual demonstration of the presence in higher plant tissue of an enzyme active in reoxidizing reduced cozymase has been reported by Okunuki (1940).

SUMMARY

1. The terminal oxidase of *Avena* coleoptile respiration appears to be cytochrome oxidase. This conclusion is based primarily on the facts that an enzyme capable of oxidizing cytochrome c is found in the coleoptile and that respiration is inhibited by both cyanide and azide, inhibitors of this enzyme.

2. The *Avena* coleoptile contains enzymes for the phosphorylation of hexose to fructose diphosphate. This phosphorylation is increased in the presence of added ATP. Pyruvate can be oxidized by the coleoptile, and it is suggested that pyruvate may be the terminal product of the initial breakdown of hexose by the *Avena* coleoptile.

3. The oxidation of pyruvate by the *Avena* coleoptile is inhibitable by malonate, and this inhibition may be partially reversed by the further addition of fumarate, isocitrate or α-ketoglutarate. In the presence of malonate, both fumarate and α-ketoglutarate are converted to, and accumulate as, succinate. These facts are in accord with the hypothesis that respiration in the *Avena* coleoptile proceeds through a system generally similar to the Krebs cycle.

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An Apparatus for the Incubation of Tissue Slices and Homogenates

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The widespread use of Warburg vessels for metabolic studies not requiring the measurement of gas exchange involves fragile and expensive glassware, necessitates separate handling of each vessel, and limits the number of experiments which may be performed at one time.

In the present paper a new apparatus[1] for the incubation of tissue slices and homogenates is described which was specifically designed for studies not involving manometric measurements. This apparatus is rugged and simple to operate. The reaction vessels are standard 20 ml. pyrex beakers held in a stainless steel tray. This tray containing as many as 30 reaction vessels is treated as a unit; all the vessels may be cooled, incubated, boiled, and gas equilibrated simultaneously. The vessel container was designed to bring the solutions within the vessels in approximate equilibrium with the bath water. As a result, the evaporation losses are negligible even during long periods of incubation, and readjustment of the solution volume at the end of the experiment is obviated. If necessary, two different gases may be passed through the apparatus at the same time.

A water bath was designed for this apparatus whose temperature is easily and quickly adjusted over a wide range or quickly brought to a boil for protein coagulation. This bath occupies only 1.5 square feet of space and may be placed in a cold room or refrigerator for incubation below room temperature. The bath and vessel rack is adaptable to a wide variety of procedures requiring temperature regulation and shaking. A general view of the apparatus is shown in Fig. 1.

[1] This apparatus may be obtained from the Precision Scientific Co., 3737 Cortland St., Chicago, Ill.

The Vessel Container

The vessel container (Fig. 2) made of 22 gauge stainless steel, consists of a gabled cover, A, which slides into and rests on the bottom of a 30-place beaker rack, B. The 3/16 inch openings at the bottom ends of the cover, D, allow the bath water to come into direct contact with the vessels while maintaining a water seal for the gas within container. The excess gas passed through inlet, I, escapes beneath the water alternately at each end of the cover as the container is shaken.
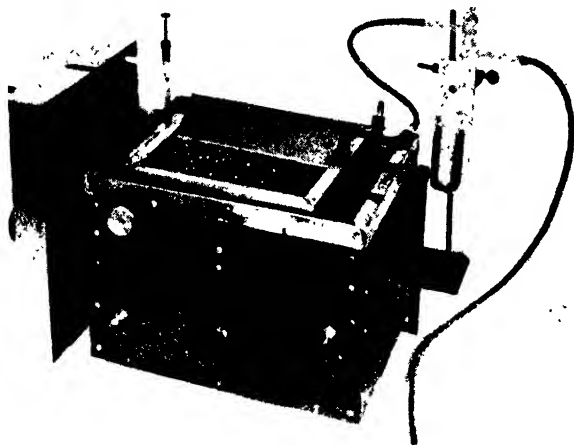
Fig. 1. View of water bath with its cover at the left. Front cover of vessel tray removed to show beakers.

Two groups of 12 vessels each may be equilibrated with two different gases within a single container by using two smaller covers shown in end view in Fig. 2, C. The end openings of these covers, E, are designed to let the gases escape at opposite sides of the container.

The beaker spacers, S, made of $1/8$ or $5/32 \times 3/4$ inch flat head stainless steel rivets[2] or rods pass through and are soldered to the bottom of the container. The distance between centers is 1 7/16 inches. A hole, O, is provided to engage the hook of the shaker arm of the water bath

[2] Stainless steel rivets may be obtained from Industrial Steels, Inc., 246 Bent Street, Cambridge 41, Massachusetts.

(Fig. 3, H). It is convenient to die stamp numbers on the bottom of the tray to correspond to the beaker numbers.

The presence of water within the container not only increases the speed of the temperature equilibration, but also saturates the gas
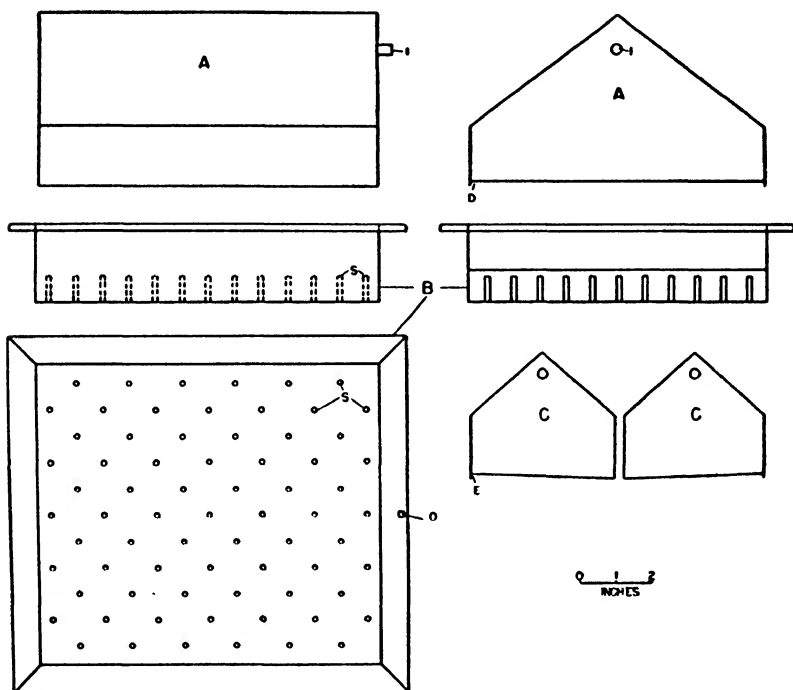
FIG. 2. Vessel container showing beaker tray and two types of covers.

phase and thereby reduces evaporation losses from the solution. Since this was a prime consideration in the design of the apparatus, the following factors influencing these losses have been studied.

  a. It is important to maintain the temperature of the gas phase within the container as close to that of the water bath as conveniently possible. This is practically accomplished by the use of a cover for the whole bath (Fig. 3, N). Without this cover the evaporation rate is increased at least three times. However, insulation of this cover or of the container cover is not necessary with normal laboratory temperatures.

  b. As the number of beakers within the container is increased and the amount of water bath surface within the container decreased, the rate of evaporation increases (Table I). In order to keep this surface at a maximum, pins of low cross-sectional area

FIG. 3. The water bath.

TABLE I

Rate of Evaporation from Vessels in Container

4 ml. of 0.9% sodium chloride in each vessel; gas flow 0.5 cu. ft./hr.

No. of vessels	Temperature °C.	Rate of evaporation
6	38	0.0050 ± 0.0016 ml./hr.
24	38	0.0075 ± 0.006 ml./hr.
30	38	0.015 ± 0.006 ml./hr.
30[a]	38	0.011 ± 0.001 ml./10 min.
24	100	0.026 ± 0.023 ml./5 min.
30	100	0.044 ± 0.034 ml./5 min.

[a] Gas flowing at the rate of 5.0 cu. ft./hr.

are used to hold the beakers in place. With this type of rack the evaporation losses are only half those with conventional types of racks.

c. The rate of evaporation of water depends to some extent on the diameter, height, and extent of flare of the beakers. Although selection for uniformity would reduce the standard deviation somewhat, it is probably not justified under the conditions described.

Losses in a boiling water bath are no greater during a 15 minute period than during a 5 minute period, the time usually employed for coagulating protein—the major loss is during cooling in both cases. Volume changes over longer periods of time are variable, and water may even be taken up by isotonic solutions.

Water Bath

The water bath shown in Fig. 3, B, is made of 22 gauge stainless steel except for the bottom, which is made of copper for greater heat conductivity. The flat heaters, H, backed by brass plates, HB, are fastened underneath the bottom by means of machine screws whose heads are soldered to the bottom of the bath. The wires, W, from the heater units are covered by asbestos or fiber glass sleeving and pass through the holes, J, to the 5 place terminal strip, TS, mounted on bracket, A, which also supports the masonite sheet, M. The space between the bath and sheet M is packed with rock wool. The bath proper is mounted on a box made of pressed masonite braced at the corners by ¾ inch angle iron, O. This box is split at Q so that the upper part may be removed to expose the shaking motor and other accessories mounted on the plywood base, E, and the lower front panel. One-half to ¾ inch openings, V, spaced around the sides and back provide ventilation for the interior.

The thermoregulator, TR (see section on heating circuits), is fastened to the side of the bath by means of the clamp, K, held by a wing nut and a machine screw soldered through the side of the bath, and extends through a plate, X, which covers this end of the bath. A Weston all-metal dial type thermometer, T, reading from 0 to 100°C. is soldered to the front of the bath.

The vessel container (Fig. 2) rests on ⅝ × ¼ inch rollers, R, which move in the ¼ inch channels, C, and is engaged by the shaker hook, H, and spring clip, S. A length of travel approximately 1.5 inches is brought about by the rocker arm, RA, actuated by the motor crank, CR, turning on a ⅜ inch radius. The water level is adjusted by means of the constant level device, CL, so that it is about ¼–⅜ inch above the bottom of the vessel container. The water should just maintain a gas seal at the ends of the container cover. If the level is too high, there is danger of floating the beakers.

Heating and Electrical Circuits (Fig. 4)

It is desirable for studies with tissue slices or extracts to have a bath whose temperature can be more easily varied than the conventional large baths and also be rapidly brought to a boil for protein coagulation. The latter condition is met by having a water volume of only 1.5 liters and a heat input of 1190 watts. Under these conditions the bath can be brought to a full boil in less than 10 minutes. For low temperature incubation a relatively high heat input of 120 watts compensates for the low heat capacity of the bath. Thus, although the temperature drops from 38° to 37°C. when a full container is placed in the bath, the temperature returns to 38°C. within 2 minutes. On the other hand, a full container which has been cooled in an ice bath requires 7 minutes

to return to incubation temperature. This delay is not disadvantageous since about the same time is necessary to completely sweep out the air from the large˟container.
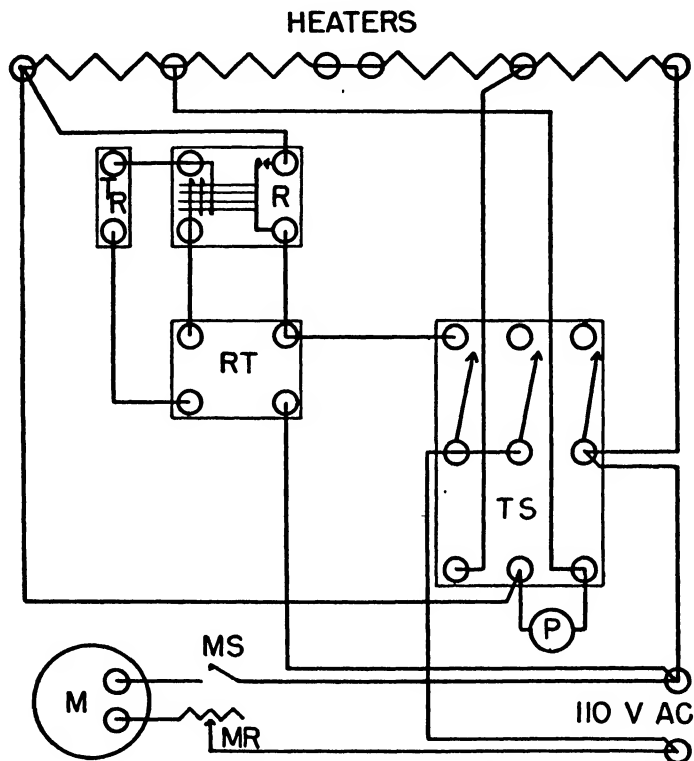
FIG. 4. Electrical circuit. H, two General Electric stove units, DS 828, 8 inch, 950 watt; TS, triple pole double throw switch, Cutler and Hammer No. 8661K1; MS, single pole motor switch; R, Struthers-Dunn 6 volt D.C. 2 amp. supersensitive relay; RT, Aminco Rectan transformer; P, 10 watt 110 volt pilot light; M, 1/80 H.P., 139 R.P.M. Bodine geared motor; MR, 50 watt 300 ohm rheostat.

The placement of the heaters beneath the copper bottom of the bath results in a uniform distribution of heat. Even without stirring the temperature does not vary by more than ±0.5°C. in all parts of the bath, and only the shaking movement of the vessel container is required to maintain the temperature within ±0.05°C.

The bath was originally designed for use with a Fenwal type thermoregulator, which is ideally suited for such a shallow bath, but this type was finally discarded in

favor of the more reliable mercury thermoregulator. The mercury reservoir of this thermoregulator is made of 10 mm. o. d. pyrex tubing 12 cm. long with an 8 cm. capillary 1.5 mm. i. d. Since the use of the bath at 100°C. forces part of the mercury to the upper part of the thermoregulator it is important to use clean mercury which will contract without a break when the bath is cooled to its regulating position. In our experience there is no hysteresis in the thermoregulator, and resetting after the bath has been boiled and cooled is, therefore, not required. Any commercially available thermoregulator which allows a large overshoot without requiring resetting may be substituted.

The location of this regulator over the heaters gives sufficient anticipation to limit the overshoot measured with a Beckman differential thermometer to less than 0.05°C. in spite of the relatively high heat input. The immediate juxtaposition of the heaters to the copper bottom reduces the heat lag to a drop of only 0.02°C. after the heating cycle begins.

The pilot light indicates by its brightness the position of the triple pole switch; it is dim in the low thermoregulated position and bright in the high position.

Mounting for a Standard Warburg Bath

The vessel containers may be shaken in a standard Warburg bath by utilizing two standard manometer supports with brackets to support the tray. A cover for the bath is recommended to reduce evaporation losses in experiments of long duration.

Gas Flow

The rate of gas flow is measured by a flowmeter having a range of 0–5 cubic feet per hour. The time necessary to completely replace the air within the container under actual experimental conditions was determined in the following manner. Commercial tank nitrogen was passed through the container at a given rate of flow. A small rubber tube led the gas from the atmosphere within through the end opening of the cover to a Pauling oxygen meter.[3] The slight pressure within the container was sufficient to maintain a continuous flow to the meter. The change in oxygen tension with time, and the time required to reduce the oxygen tension within the limit of error of the instrument to that of the incoming mitrogen is shown in Fig. 5. The recorded times give a margin of safety of about 2 minutes because of the lag of the meter.

[3] The Pauling Type C Oxygen Meter was kindly loaned to us by James P. Munn of Arnold O. Beckman, Inc., Pasadena 2, California. This instrument can be read to less than 0.0005 atmospheres of oxygen.
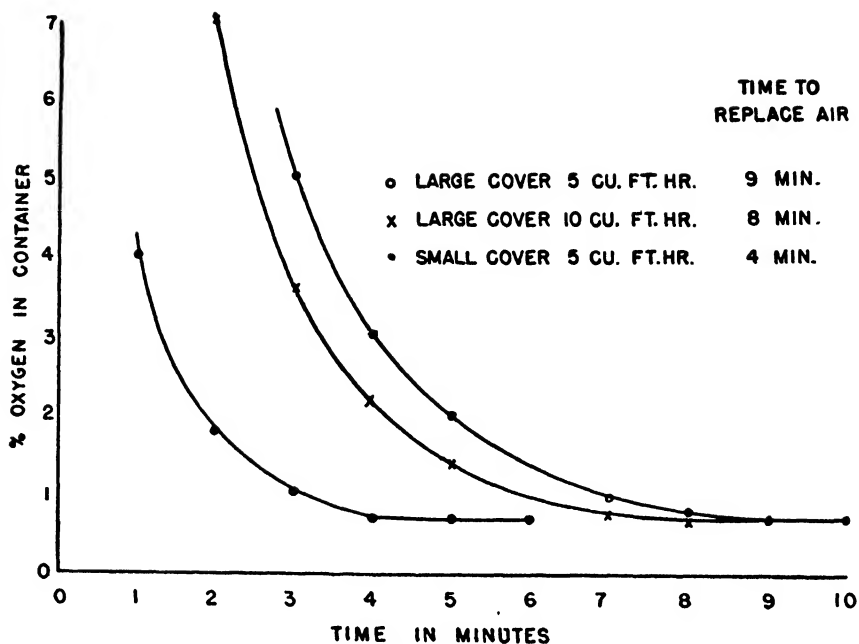
FIG. 5. Rate of replacement of air within container.

The Use of the Apparatus

The covered vessel container is placed in the bath with its flanges over the rollers. The water level should be about ⅛ inch above the container cover opening. The shaker arm and gas tube are connected to the container, and the gas passed through at the rate of 5 cubic feet per hour. As soon as the gas is seen to escape beneath the water at the end of the container, the water bath cover is replaced, with the gas tube passing through the back opening (Fig. 3, U), and the shaker motor started. The rate of shaking usually employed is about 90 oscillations per minute. After 9 minutes with the large cover, or 4 minutes with the small cover, the gas flow is reduced to 0.5 cubic feet per hour for the duration of the experiment.

At the end of the experiment the container is removed from the bath; its cover is slowly lifted up, tilted toward one end, and then drawn away in such a manner that condensed water droplets will not fall into the beakers. The reaction may be stopped by any suitable protein

precipitant. However, heat coagulation is preferred in this laboratory since it gives a protein-free filtrate uncontaminated by any reagent.

If the proteins are to be coagulated by heat, the bath is set to boil. Water is added to bring the solutions to any desired volume, and then sufficient 0.5 N HCl to bring the pH to 5. The pH adjustment is made directly in the beakers with a small glass electrode assembly. The vessels in their covered tray are now shaken in the boiling water bath with the bath cover in place. After 5 minutes the container is removed, cooled in a shallow tray of water, and the solutions filtered.

The design of the apparatus limits its usefulness in studies involving volatile reagents. Nevertheless, it may be used for the qualitative determination of the cyanide sensitivity of enzyme systems since in the case of this reagent at least the "volatility constant" is low (1, 2). For more nearly quantitative determinations all the cyanide-treated solutions may be placed under one of the small covers and the incoming gas equilibrated with cyanide by first passing it through a solution having an appropriate cyanide concentration.

The apparatus described in this paper has been used exclusively for all the metabolic studies undertaken in this laboratory in the past 4 years and has been reliable and trouble free. As many as 60 vessels have been set up at one time. The number of vessels employed in a single experiment is no longer limited by the incubation procedure but by such operations as slicing of tissue, analysis, *etc.*

SUMMARY

1. A simple, rugged apparatus has been described for incubation of tissue slices and homogenates.

2. Standard pyrex beakers are employed as reaction vessels. These are inexpensive, less fragile, and more easily cleaned than the Warburg vessels usually employed.

3. A stainless steel tray has been described which holds up to 30 reaction vessels.

4. These vessels are equilibrated with gas and brought to temperature as a single unit, resulting in complete uniformity of treatment.

5. The design of the apparatus reduces evaporation losses, and readjustment of solution volume is not necessary even after long periods of incubation.

6. The handling of the apparatus is so simple and uniform that the

size of an experiment is no longer limited by the incubation procedure; as many as 30–60 vessels may be processed with ease.

7. A compact portable water bath whose temperature may be accurately controlled over a wide range or rapidly brought to a boil for protein coagulation has been designed for use with this apparatus.

8. By means of an adapter two trays containing 30 vessels each may be incubated in a standard Warburg water bath.

9. The apparatus may be employed for analytical determinations requiring incubation or boiling.

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Relative Biological Activity of Beta-Carotene and Vitamin A [1]

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INTRODUCTION

The association of vitamin A activity with the carotenoid pigments was first observed by Steenbock (1). This observation was confirmed by v. Euler *et al.* (2) and by Moore (3), who found that carotene prepared from carrot roots possessed vitamin A activity. It was not, however, until Karrer *et al.* (4) and Kuhn *et al.* (5) demonstrated the structural relationship between β-carotene and vitamin A that a hypothesis was proposed concerning the relative biological potencies of the two compounds. Karrer postulated that, in the conversion of β-carotene into vitamin A in the animal body, the cleavage of the β-carotene molecule took place symmetrically giving rise to two molecules of vitamin A. This view was generally accepted until Holmes and Corbet (6, 7) prepared crystalline vitamin A alcohol which was found to have a biological potency of approximately 3,000,000 I. U./g. This was confirmed by Mead, Underhill and Coward (8). Later, Baxter and Robeson (9, 10) reported that crystalline vitamin A alcohol prepared by them had a potency of 4,300,000 I. U./g. Since β-carotene contains by definition approximately 1,667,000 I. U./g., it appeared that vitamin A was approximately twice as active biologically as β-carotene. Mead *et al.* (8) concluded either that β-carotene is not converted quantitatively into vitamin A in the animal body or that the molecule of β-carotene gives rise to only one molecule of vitamin A, the other part of the molecule having no activity. These workers favored the latter explanation.

The beneficial effect of vitamin E on utilization of vitamin A was first observed by Moore (11, 12) and later by Sherman (13), Quackenbush *et al.* (14), and many others whose work is reviewed by Hickman (15) and Lemley *et al.* (16).

Data presented by Hickman (15) indicated that tocopherol had a greater synergistic effect on β-carotene than it had on vitamin A alcohol, and concluded that β-carotene was more than half as active as the free vitamin. He suggested that the conversion of β-carotene into vitamin A *in vivo* is by fission rather than by an oxidation reaction destructive to one end of the molecule. Hickman *et al.* (17, 18) and Harris *et al.* (19) concluded that the optimum level of tocopherols necessary to enhance vitamin A activity is greater for β-carotene than it is for vitamin A alcohol or acetate.

In the light of the conflicting results cited above, the present investigation was undertaken to determine the relative biological potencies of pure β-carotene, vitamin A alcohol and acetate for the rat under identical dietary conditions in the presence of adequate α-tocopherol.

EXPERIMENTAL PROCEDURE

Vitamin A Sources

The β-carotene used in these studies was isolated from sweet potatoes as described previously by Sherman and Koehn (20). It had an $E_{1cm}^{1\%}$ value of 2540 at 450 mμ and 2220 at 478 mμ in hexane as determined with a Beckman spectrophotometer. In comparison with the absorption coefficients given by Zscheile et al. (21) for pure β-carotene, it may be calculated that the β-carotene used in the present investigation was 98% pure. Chromatographic analysis (MgO) revealed the presence of 0.24% of a highly adsorbed pigment. No α- or γ-carotene was present. The remainder of the impurity (1.76%) was colorless, and was probably composed of sterols. The concentration of β-carotene in the solutions used for feeding was determined spectrophotometrically on the basis of the absorption coefficients of Zscheile et al. (21).

The vitamin A alcohol and acetate were purchased from Distillation Products, Inc., and were found to have $E_{1cm}^{1\%}$ 328 mμ values of 1750 and 1550, respectively, in ethanol as determined with a Beckman quartz spectrophotometer and L values of 4235 and 3760, respectively, as determined by the technique described by Koehn and Sherman (22).

Preparation of Supplements for Feeding

The β-carotene, vitamin A alcohol and acetate were dissolved in n-hexane[2] in such concentrations that 0.10 ml. contained the desired daily dose. To each solution sufficient α-tocopherol (Merck) was added to furnish 1.0 mg. daily per rat. The solutions were stored in the dark at 1°C. The potency of these solutions was checked weekly by means of the spectrophotometer and the Carr-Price reaction. The β-carotene and vitamin A acetate solutions showed no deterioration during the 6-week test period. The vitamin A alcohol solutions deteriorated 5.7% in one week, and, therefore, fresh solutions were prepared each week, and the daily dosage was adjusted to compensate for this loss.

Bioassay

Rats used for these experiments were taken from stock females (Alabama Agricultural Experiment Station strain) that had been fed the regular stock diet used in this laboratory (Table I) supplemented with whole milk. When the litters were born, the females were changed from the stock diet to diet 40A (Table I) supplemented with a mixture composed of 2 parts of skim milk powder and 1 part of hydrogenated cotton-

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[2] The n-hexane (b. p. 68°–71°C., sp. gr. 0.6846) used in this study was donated by the American Mineral Spirits Co. of New York.

TABLE I
Basal Diets

Ingredients	Stock diet 40 g.	Diet 40A g.
Ground wheat	645	595
Meat scrap	100	100
Casein	120	100
Skim milk powder	—	100
Alfalfa leaf meal	20	—
Molasses, blackstrap	50	50
Lard	50	—
Hydrogenated cottonseed oil	—	50
Cod liver oil	10	—
Salt, iodized	5	5

	Diet 46A[a] g.	Diet 46A2[a] g.
Sucrose	758	778
Casein, alcohol extracted	180	180
Salt mixture No. 5[b]	40	40
Cottonseed oil	20	—
Choline chloride	2	2
α-Tocopherol (Merck)	0.10	—

[a] The following amounts of vitamins were added to these diets expressed in mg./kg.: niacin 20, riboflavin 4, thiamine 2, pyridoxine 2, Ca pantothenate 10, i-inositol 200, and calciferol 0.125.

[b] Salmon, W. D., *J. Nutrition* **33**, 155 (1947).

seed oil *ad libitum* instead of whole milk. When the young rats reached a weight of 40–50 g. (23–26 days), they were transferred to individual metal cages and fed the depletion diet 46A (Table I) at the restricted level of 4 g. daily. At the end of 5 weeks, or earlier in rare instances when symptoms of xerophthalmia appeared before this time, the rats were fed diet 46A *ad libitum* and were weighed daily until growth ceased. Usually by the third day the temporary growth caused by the increased food consumption ceased, indicating depletion of vitamin A stores.

It was found that if the rats were fed diet 46A *ad libitum* during the entire depletion period, the length of time required for depletion and the weight of the rats at depletion varied considerably. Moreover, the rats reached an average body weight of 140 g. at depletion, which did not allow maximum response to the supplements in the subsequent test period. As a result of feeding trials with various restricted levels of food intake during the depletion period, the 4 g. level was selected as giving the most uniform results. At this level the rats were depleted at much lower body weights, which allowed a greater growth response to the supplements.

The depleted rats (65–90 g. body weight) were changed to diet 46A2 (Table I) *ad libitum*, supplemented with 0.10 g. refined cottonseed oil daily. Each litter, usually 8 in number, was then divided in half with equal sex distribution; one-half the litter was fed the β-carotene supplement and the other half the vitamin A supplement. The basal diet was fed in the afternoon and the feed jars were removed early the next

morning. The supplements, in hexane solution, were then pipetted onto 2 g. of basal diet in a separate jar. The contents of the jar were stirred with a spatula and allowed to stand for 10 minutes for evaporation of the hexane. The supplement jars were then placed in the cages, and the supplements were usually consumed within 15 minutes. It has been shown previously in this laboratory by Sherman (23) that destruction of supplements by this procedure is negligible, particularly since they were stabilized with α-tocopherol. Vitamin A alcohol supplements mixed with the basal ration showed no deterioration in 2 hours. The supplements were fed daily and the rats were weighed weekly during the test period.

RESULTS

The results of these experiments are presented in Table II. In every experimental group, within which sex and litter distribution was strictly practiced, it is evident that β-carotene was equally as active for the rat as vitamin A alcohol or acetate. Results from groups 1 and 3 indicate that 1.0 γ of β-carotene was definitely more active than 0.75 γ of vitamin A alcohol, whereas, results from group 4 indicate that 1.0 γ of vitamin A alcohol was more active than 0.75 γ of β-carotene. The data obtained from groups 2, 5, and 6 indicate that β-carotene and vitamin A alcohol had equal activities. Similar results were obtained by feeding β-carotene and vitamin A acetate at stoichiometrically equivalent levels (groups 7, 8). In interpreting these data, it must be borne in mind that litter and sex distribution was uniform within each group, but varied from group to group so that comparisons between groups are not valid.

DISCUSSION

The confusion existing in the literature as to the relative potency of β-carotene and vitamin A has resulted from the use of diets deficient in one or more dietary essentials in addition to vitamin A and from the use of impure preparations of β-carotene. Zscheile et al. (21) and Sherman and Koehn (20) have pointed out the difficulties encountered in preparing pure β-carotene. Many investigators have used commercial crystalline carotene stated to contain 90% β- and 10% α-carotene. Spectrophotometric examination in this laboratory of such samples of carotene have shown that in addition to α-carotene, 10–28% of colorless impurities was present. Thus, these preparations would be expected to be only 68–85% as active as the β-carotene used in the present study which contained 2% of impurity for which correction was made in feeding.

TABLE II

Growth of Vitamin A-Depleted Rats Receiving Various Supplements

Group no.	Daily supplement[a]	No. of rats	Initial weight	Gain at end of					
				1st wk.	2nd wk.	3rd wk.	4th wk.	5th wk.	6th wk.
1	0.50 γ Vitamin A alcohol	9	83	9	16	20	25	23	21
	0.75 γ Vitamin A alcohol	9	80	14	22	28	40	50	57
	0.75 γ β-Carotene	9	80	14	27	36	54	67	76
	1.00 γ β-Carotene	9	83	15	32	45	62	84	90
2	1.00 γ Vitamin A alcohol	22	81	15	34	46	59	70	83
	1.00 γ β-Carotene	22	82	14	32	45	56	68	80
3	1.00 γ Vitamin A alcohol	10	66	17	33	50	66	81	92
	0.75 γ β-Carotene	10	65	15	32	45	57	71	80
4	0.75 γ Vitamin A alcohol	14	89	9	22	35	40	50	56
	1.00 γ β-Carotene	14	89	16	33	47	60	72	84
5	0.75 γ Vitamin A alcohol	12	74	14	30	47	61	73	82
	0.75 γ β-Carotene	12	72	18	36	55	68	78	88
6	1.25 γ Vitamin A alcohol	11	80	12	27	40	64	78	94
	1.25 γ β-Carotene	11	79	12	27	43	62	81	97
7	1.15 γ Vitamin A acetate	10	74	13	31	45	58	75	87
	1.00 γ β-Carotene	10	72	15	34	50	63	76	89
8	2.30 γ Vitamin A acetate	11	79	21	45	65	87	109	125
	2.00 γ β-Carotene	11	79	19	42	61	84	104	116
9	No supplement	16	78	− 6	− 9	−16	—	—	—

[a] Each rat received 1.0 mg. of α-tocopherol daily which was contained in the supplement.

The experiments herein reported were designed to determine the relative biological activity of β-carotene and vitamin A alcohol and acetate for the rat under carefully controlled dietary conditions. For this purpose highly purified diets were used and adequate amounts of all known dietary essentials including α-tocopherol were supplied in pure form.

The data presented indicate that, under these conditions, β-carotene was quantitatively converted into vitamin A in the rat. This lends support to the theory that the conversion of β-carotene into vitamin A *in vivo* is by fission of the carotene molecule to form two molecules of vitamin A and not by an oxidative reaction that inactivates one-half of the carotene molecule.

It is interesting to compare these results with those obtained by other workers recently. Deuel *et al.* (24) found that the response of vitamin A-depleted rats to β-carotene administered simultaneously with α-tocopherol was markedly greater than that obtained previously with similar unitage of reference cod liver oil, and suggested that the potency of 0.6 γ of β-carotene far exceeds the biological activity of one USP unit of reference cod liver oil.

Porter *et al.* (25) showed that the growth response of vitamin A-depleted rats receiving β-carotene prepared from tomatoes, and optimum amounts of α-tocopherol far exceeded that expected when compared with the response to the USP reference cod liver oil. They concluded that the USP reference oil contains less vitamin A than it is stated to contain.

Callison *et al.* (26) found that, in assaying carrots, 30–44% less vitamin A activity was found when β-carotene was used as the standard than when the USP reference oil was used.

Hickman (15) and Lemley *et al.* (16) pointed out that the USP method for vitamin A assay gives variable and inaccurate results because the USP test diet contains insufficient and variable amounts of tocopherols. Since β-carotene requires more tocopherol for optimum growth response than vitamin A, the USP method produces results that indicate that the ratio of potencies of vitamin A alcohol to β-carotene is greater than it actually is.

Sherman (27) presented data, obtained with the same preparation of β-carotene as used in the present study, that showed 2 γ of β-carotene to be markedly superior to 1 γ of vitamin A or 1.15 γ of vitamin A acetate in producing growth response in vitamin A-depleted rats receiving a diet containing lard and supplemented with α-tocopherol. Although he attributed the superiority of the β-carotene over vitamin A to greater gastro-intestinal stability in the presence of lard, it may be attributed equally well to the fact that the level of β-carotene fed was twice as great as that of vitamin A.

The evidence that has accumulated indicates two facts: (1) that the

vitamin A activity of β-carotene has been generally underestimated, and (2) that the USP reference oil has not contained the stated amount of vitamin A.

It appears, therefore, that data in the literature on the vitamin A content of foodstuffs based on bioassay with rats and expressed in terms of International Units and USP units of vitamin A are of little value. Not only are they based on inaccurate standards, but they were obtained by methods that have been shown to be unreliable.

The results presented in this paper indicate the need for a revision of current methods of bioassay in which the proper attention is given to the use of an accurate primary standard and of an adequate test diet. The role of tocopherol in this diet should be given special consideration.

SUMMARY

Data presented indicate that β-carotene when fed to rats with adequate α-tocopherol is converted quantitatively into vitamin A. Support is given to the theory that conversion of β-carotene into Vitamin A *in vivo* is by fission of the molecule, yielding two molecules of vitamin A, and not by an oxidation reaction that inactivates one-half of the molecule. These results are discussed in relation to the I. U. and USP unit of vitamin A.

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On the Mechanism of Enzyme Action. XXXIII. Fat Formation in Fusaria in the Presence of a Pigment Obtained from *Fusarium solani* D₂ Purple

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INTRODUCTION

The observation was made that when *Fusarium lycopersici* (Flyco) and *Fusarium solani* D₂ *purple* (FsD) were grown on sugar concentrations ranging from 2.5 to 10%, there was an increase in both fat and pigment production. It was conceivable then that there could be a significant change in the correlation between fat formation and carbohydrate utilization in the presence of the pigment formed and, therefore, the phenomenon described above was subjected to a detailed study.

Since both Flyco and FsD can be obtained pigmented, it was of interest to investigate what effect, if any, a pigment isolated from FsD may have on the enzymatic activity of a non-pigment producer, such as *Fusarium lini Bolley* (FlB).

That pigments isolated from molds and bacteria influence such actions has been shown by Nord and collaborators (1), Hoffmann-Ostenhof and co-workers (2) and others. Therefore, with the FsD pigment, the structure of which was recently described elsewhere (3), exploratory experiments were carried out as to its possible influence on the rate of dehydrogenation of isopropanol by FlB.

EXPERIMENTAL

I. Experiments on Acetone Formation

Fusarium lini Bolley No. 5140 obtained from the *Biologische Reichsanstalt*, Berlin-Dahlem, through the courtesy of Dr. H. Wollenweber before the outbreak of hos-

tilities, was used in these experiments. The stock cultures were maintained on the following medium:

> 20.00 g. agar
> 40.00 g. glucose
> 5.00 g. potassium nitrate
> 5.00 g. potassium phosphate (primary)
> 0.75 g. magnesium sulfate (7H₂O)
> 1000 ml. tap water

and periodically examined to check their purity.

Cultural Conditions

For the experiments, a salt solution consisting of the following:

> 5.00 g. potassium nitrate
> 5.00 g. potassium phosphate (primary)
> 0.75 g. magnesium sulfate (7H₂O)
> 1000 ml. tap water

was sterilized at 120°C. under 15 pounds pressure for 20 minutes. The various concentrations of pigment used were dissolved in the isopropanol and these were added with aseptic technique to the sterilized salt solution. The concentration of the alcohol in the medium was approximately 0.8% and the initial pH 4.2. In all these experiments, the FlB was grown in 125 ml. Erlenmeyer flasks containing 50 ml. of salt solution. Inoculation was made by means of a spore-mycelial suspension of cultures grown on the stock nutrient medium containing 2% agar.

Analytical Methods

Four flasks were removed on the respective day of determinations and the combined filtrate analyzed (4) in duplicate for acetone and isopropanol (1), as heretofore. After the 21st day, the mycelial weights were determined by filtering, drying, and weighing the mycelium from four flasks.

RESULTS AND DISCUSSION

The results are recorded in Table I.

It will be noticed that the control reaches the maximum acetone formation on the 14th day, while those flasks containing the pigment attain their highest values on the 16th day. Also, with the 4 lowest concentrations of pigment, e.g., from 12 to 87γ/50 ml., the amount of acetone reached at the maxima is approximately that of the control, indicating that, although the progress of dehydrogenation was delayed by 48 hours, there was no inhibition of the growth of the mold. At the highest concentration, however, e.g., 145 γ/50 ml., the maximum value is still reached 48 hours later than the control, but there is a slight inhibitory effect as shown by comparison with the highest amount of acetone obtained. Thus, the pigment, in low concentrations, diminishes

TABLE I

Dehydrogenation of Isopropanol by Fusarium lini Bolley (FlB)

Quantity of F₃D pigment (γ/50 ml.)	Acetone (mgs./50 ml.)					Mycelial weight after 21 days (mg.)
	8th day	10th day	14th day	16th day	18th day	
0 (Control)	19.7	23.5	46.5	30.6	11.7	29.4
12	21.3	24.9	41.0	53.8	33.5	19.4
29	20.7	21.9	38.1	52.1	34.2	20.5
58	19.8	22.9	41.4	51.8	40.6	26.0
87	19.5	20.0	36.7	48.8	33.6	27.8
145	17.6	18.1	33.8	39.9	21.1	28.4

the rate of this dehydrogenation by about 12 percent without exerting an inhibitory action, as shown by the fact that the mycelial weights are approximately the same.

II. Influence of the Pigment on the Carbohydrate Conversion Factor

Since the pigment influenced the rate of dehydrogenation by a non-pigment producer, as shown above, it was decided to use the pigment at various concentration levels in glucose solutions of different concentrations, again with FlB as the test organism, and to investigate the effect this pigment may have on fat and sterol production, as well as on the carbohydrate conversion factor.

EXPERIMENTAL

The same FlB cultures described previously were utilized. For the experiments, a Raulin-Thom salt solution consisting of the following:

4.00 g. tartaric acid
4.00 g. ammonium tartrate
0.60 g. potassium carbonate
0.60 g. dibasic ammonium phosphate
0.40 g. magnesium carbonate
0.25 g. ammonium sulfate
0.07 g. zinc sulfate ($7H_2O$)
0.07 g. ferrous sulfate ($7H_2O$)
1500 ml. tap water

was prepared, and to it was added glucose to the extent that the final concentration of the carbohydrate amounted to 2.5, 5 or 10%, respectively. The pigment was dis-

solved in acetone and added to the medium before sterilization. The mold was grown in 3-l. Fernbach flasks, one liter of solution being added to each flask. All series were set up in triplicate. The flasks were inoculated with a 5 ml. spore mycelial suspension. After 3 weeks, the media were filtered and the contents and mycelia of each series combined. The solution was analyzed for sugar by the Munson-Walker procedure (5), and the fat and sterol determinations were carried out as previously described (6).

A few words should be said about the measurement of the total pigment production. It has been noticed that FsD produces several pigments besides the one isolated and studied (3). For example, there is a pigment present in the mycelium which is not identical with the one isolated, but which also changes color with alkali. Pigment determinations were carried out both on the mycelia and medium. The pure pigment isolated from FsD was used as the standard. It was found that maximum absorption of the violet color was at 565 mμ, and that the Beer-Lambert law was followed up to 9 γ/ml. of test solution.

The determinations proceed as follows: An aliquot of the medium containing the pigment (after removal of the mycelium) was brought up to 95 ml. with water and 5 ml. of 1 N sodium hydroxide solution added. The solution was mixed thoroughly and read immediately in an Evelyn photoelectric colorimeter, using as a blank 95 ml. of water and 5 ml. of 1 N sodium hydroxide solution. The color obtained from the solution is stable for at least 5 minutes. Pigment determinations on the mycelium were carried out by taking an aliquot of the chloroform extract used for total lipide and sterol determinations, evaporated to dryness and dissolved in 2 ml. of acetone. The solution was then diluted with water to 95 ml., and the test carried out as described above. Turbidities resulted due to the fat present, and this decreased the stability of the color, so that with these aliquots, the readings must be taken within one minute after the addition of the sodium hydroxide solution. Corrections for the turbidities present were made. Since there are present other pigments which change their color similarly to that of the identified FsD pigment, the determinations will measure all of these compounds.

DISCUSSION

The results are presented in Table II.

The following facts are to be pointed out. On a 2.5% solution of glucose, with 1, 2, and 4 mgs. of pigment added, there is a decrease of 31, 47, and 58%, respectively, in mycelial weights as compared with the controls (see Table IV), while with the 5% solution, there is a decrease of 38, 40, and 45%, respectively, in mycelial weights. With 10% glucose solution, there is a decrease of 13, 37, and 42% as the pigment level is increased. From the values recorded, it can be noticed that, in all cases, the greater the concentration of pigment, the greater the decrease in mycelial weights when compared with the controls. This reduction in mycelial weights is comparable with the results obtained by Hoffmann-Ostenhof (2), who found that various naphthoquinone

TABLE II

Effects of FsD Pigment on Fat Formation in Fusarium lini Bolley (FlB)

Glucose in medium	Pigment in medium	Average mat weight	Unutilized glucose	Total lipide	Sterol	Fat coefficient[a] (Carbohydrate conversion factor)
g./l.	*mg.*	*g.*	*g./l.*	*Per cent*	*Per cent*	
	1	2.54	0.00	12.8	1.47	1.30
25	2	2.28	0.00	9.8	1.00	0.89
	4	1.80	0.20	6.4	0.75	0.47
	1	2.67	2.18	6.3	0.65	0.35
50	2	2.59	3.20	6.1	0.65	0.34
	4	2.39	6.10	6.7	0.75	0.37
	1	3.84	22.32	4.9	0.60	0.21
100	2	2.79	21.60	4.4	0.64	0.16
	4	2.58	25.23	4.4	0.64	0.15

[a] The fat coefficient is defined as the number of grams of total lipide produced/ 100 g. of carbohydrate consumed.

derivatives, in low concentrations, decreased the growth of yeast by about 50%. It is to be mentioned here that the greatest differences occur with the 2.5% glucose solutions, at which level maximum fat formation occurs in the control.

The greater the concentration of pigment in a series, the more the amount of sugar consumed by the mold decreases, *e.g.*, on the 5% glucose solution, with 1 mg. of pigment, 2 g. of sugar remain unutilized, while with 4 mg. of pigment, 6 g. of sugar remain.

Within the 5 and 10% series, with all concentrations of pigment, the amount of fat and sterol formed is about the same. The amount of fat formed in these two series is the same as in the controls (Table IV, Raulin-Thom) but the carbohydrate conversion factor for the 5% solution is only 52% of the control, while with the 10% solution, it is 72% of the control. Thus, more glucose is used to produce a certain quantity of fat, and consequently the pigment has influenced the utilization and conversion of the carbohydrate by the mold, suggesting that it may act on one or more enzyme systems as an inhibitor. However, in the case of the 2.5% series, it was observed that the pigment may act both as an accelerator and as an inhibitor. When 1 mg. of

pigment is added, the fat formed is increased 24% over that of the control, while with 4 mgs., there is a decrease of 34%. But in all cases, the carbohydrate conversion factor is still lower than in the control.

It has been indicated (2, 7, 8, 9) that quinones and their derivatives are capable of inhibiting enzymes, the carriers of which contain -SH groups, and since these may be present in our respiratory and dehydrogenating systems, an action on these could account for the effect of the pigment. The lowest concentration of quinones used by other investigators was 10^{-5} M. However, the lowest concentration of FsD pigment (M.W. = 290) used in these experiments at which the acceleration occurs, is 10^{-6} moles, or a concentration 10 times lower than that used in experiments on yeasts (2). Thus, the threshold level at which the FsD pigment causes a measurable inhibition of these enzyme systems may not be reached at the lowest concentration, and thus there is no noticeable inhibitory effect. At this concentration our pigment may very well have a beneficial action on *another* enzyme system present.

On the 2.5% glucose series, there is an increase of 50% in sterol formation over that of the control at the 1 mg. level of pigment, and thus the pigment, here too, as in the case of the fat, has an accelerating effect on the carbohydrate conversion. However, with the 2 and 4 mg. concentrations of pigment, the amount of sterol is lower than in the control, being 25% less on the 4 mg.-containing media. It may be that with the concentration of 1 mg. of pigment, in which there is a beneficial effect, the pigment acts as an accelerator on some of the enzyme systems which are interacting in the formation of the common acetaldehyde and/or acetic acid used in the synthesis of both fat and sterol. All in all, these experiments demonstrate that, depending on its concentration, the pigment exerts both an accelerative and inhibitory effect on fat and sterol production as well as on the carbohydrate conversion factor.

Since it is known that quinones may also inhibit proteolytic enzymes, macro Kjeldahl nitrogen determinations were carried out on samples of mycelia obtained in these experiments. These results are recorded in Table III.

It will be noticed that there is a decrease in the amount of nitrogen present as compared with the contents of the control, the greatest effect, here too, being observed on the mats grown on the 2.5% glucose solutions.

TABLE III

Effects of FsD Pigment on the Nitrogen Metabolism of FlB

Sample no.	Glucose	Pigment in medium	Nitrogen	Difference from control
	Per cent	*mg./l.*	*Per cent*	*Per cent*
1	10	1	6.03	−7
2	10	4	5.82	−10
3	5	2	5.78	−11
4	2.5	1	5.17	−20
5	2.5 (control)	0	6.47	0

III. Effects of Various Media on the Growth of FlB, Flyco and FsD

It was also thought interesting to study the effects on the fat and sterol production as well as the fat coefficient caused by varying the composition of the media normally used for growing FlB, Flyco, and FsD in this laboratory. The normal medium for Flyco[2] and FlB is Raulin-Thom, while that for the FsD is Czapek-Dox. All experiments were run on a 3 week basis, unless otherwise specified, with the same cultures of FsD and Flyco as were used heretofore (6). In Table IV are recorded the results obtained when FlB is grown on the Raulin-Thom (control) and the modified Czapek-Dox (6). Comparing the fat and sterol values, and the carbohydrate conversion factors, it will be noticed that there is no difference in fat and sterol formation with increasing concentrations of glucose when grown on the Czapek-Dox in relation to

[2] In the course of these studies, other investigations in our laboratory necessitated establishing conditions under which one of these organisms would give us large quantities of fat devoid of pigment. The literature revealed the claim (10) that Flyco, when grown on the 'M' solution of Lockwood (11), forms 36% fat measured on a dry weight basis.

We grew Flyco on the stated medium (containing 30% glucose) for 8 weeks at 28–30°C. At the end of this time, the mycelia were filtered off, washed with running distilled water and air-dried. After removal of the mycelium, the filtrate was tested for the presence of reducing substances, using Fehling's solution, and was found to be positive. Fat determinations of this dry mycelium by a previously described method (6), and also by the one mentioned above (10), were carried out.

To establish the efficiency of the first method as compared with the second, parallel quantitative estimations were performed on FlB which had been grown for 3 weeks on a Raulin-Thom medium (containing 2.5% glucose) at 28–30°C. Since it is known that sugars are slightly soluble in an alcohol-ether mixture, the suitability of the second method was tested by determining quantitatively the amount of water-

TABLE IV

Fusarium lini Bolley (FlB) Grown for Three Weeks

Type medium	Glucose in medium	Average mat weight	Total lipide	Sterol	Unutilized glucose	Fat co-efficient
	g./l.	*g.*	*Per cent*	*Per cent*	*g./l.*	
Raulin-	25	4.28	9.7	0.99	0.00	1.61
Thom	50	4.33	7.3	0.74	3.5	0.67
(control)	100	4.43	4.1	0.65	27.2	0.25
	25[a]	3.95	8.5	0.93	0.00	0.84
Czapek-Dox	50	5.05	7.3	0.79	1.4	0.76
	100	4.66	4.6	0.56	17.6	0.26

[a] On 2.5% glucose, an orange pigment was excreted into the medium.

the control, but that the fat coefficient is 50% lower than in the 2.5% glucose solution. Furthermore, an interesting observation was made when FlB was grown on the Czapek-Dox medium containing 2.5% glucose. An orange colored pigment which is insoluble in organic solvents from both acid and alkaline solutions, and whose color changes

soluble and ether-soluble substances in the alcohol-ether extract. These results are presented in the following table:

Fat Formed in Flyco and FlB
(Before and after lyophilization)

Organism	Unlyophilized	Lyophilized			
	% Total Lipide by CHCl₃ extraction[a]	% Alcohol-ether sol. substances[a]	% Ether-sol. substances[b]	% Water sol. substances[b]	% Actual tot. Lipides[a]
FLYCO	3.5	66.0	16.0	84.0 ·	11.0
FlB	12.0	15.0 ·	90.0	10.0	12.5

[a] Based on dry mat weight.   [b] Based on dry weight of alcohol-ether sol. residue.

From the recorded data it is obvious that our results are at variance with those obtained by Stotz, and that the *actual* amount of extractable fat in both organisms is approximately the same when grown under the conditions stated. The removal of lipides from Flyco is more difficult than from FlB due to the presence of excess amounts of unused sugar 'trapped' within the mycelium thereby masking the fat, as shown by its *incomplete* removal by chloroform extraction, and the complete removal by alcohol-ether extraction.

from orange (alkali) to red (acid), was excreted into the medium. This is the first time that the formation of such a pigment has been observed when growing FlB.

Thus, changing the medium didn't cause any significant variations in the case of FlB. However, Flyco, when grown on Czapek-Dox medium instead of Raulin-Thom gives rise to significant changes (Table V).

TABLE V

Fusarium lycopersici (Flyco) Grown for Three Weeks

Type medium	Glucose in medium	Average mat weight	Total lipide	Sterol	Unutilized glucose	Fat co-efficient
	g./l.	*g.*	*Per cent*	*Per cent*	*g./l.*	
Raulin-Thom	25	9.83	4.9	0.49	0.00	1.93
	50	12.50	17.2	0.56	0.5	4.35
	100	13.32	19.4	0.56	13.8	3.00
Czapek-Dox	25	4.07	11.3	0.65	0.00	1.84
	50[a]	5.37	8.3	0.69	2.9	0.95
	100	5.13	8.2	0.63	24.8	0.56

[a] See note at bottom of Table IV.

When Flyco is grown on Raulin-Thom medium, the fat produced mounts with increasing concentrations of sugar, while on the Czapek-Dox medium, an inverse relationship is found; *e.g.*, on increasing the sugar concentration, the amount of fat decreases. This is more vividly borne out in Fig. 1. On the Raulin-Thom medium, a pigment was produced, while on the Czapek-Dox medium no pigment was obtained. This could imply that the pigment affects the enzyme systems involved in the carbohydrate conversion, for where the amount of pigment was greatest, the quantity of fat formed was the highest, while, on the Czapek-Dox medium, with no pigment formation visible, Flyco follows the same pattern as FlB. (Both on the Raulin-Thom and Czapek-Dox.)

In Table VI are recorded the results obtained with FsD grown on Raulin-Thom and Czapek-Dox media. In both cases a pigment is produced. It will be noticed that the pH on a 2.5% glucose solution (Raulin-Thom) has increased, while on the 5 and 10%, the pH has decreased. This is the first time that a decrease in pH has been observed when FsD was grown in this laboratory. This may indicate an
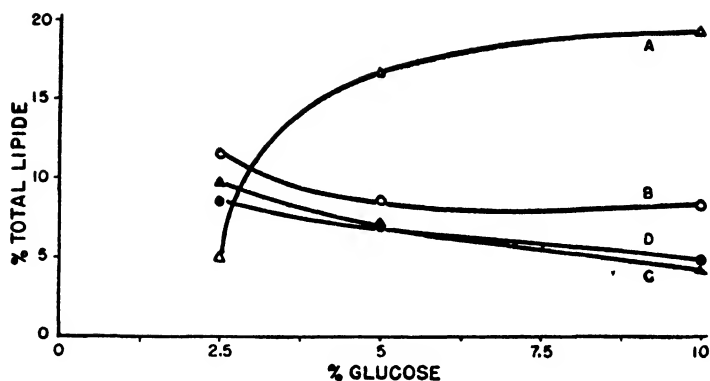
Fig. 1. Total lipide production of Flyco and FlB when grown for 3 wks. on Raulin-Thom (R-T) and Czapek-Dox (C-D) media employing various cons. of glucose.

A△ Flyco on R-T. Pigment production increased with glucose conc.
B○ Flyco on C-D. No pigment production.
C▲ FlB on R-T. No pigment production.
D● FlB on C-D. No pigment production.

TABLE VI

Fusarium solani D_2 Purple (FsD) Grown for Three Weeks

Type medium	Glucose in medium	Final pH	Average mat weight	Total lipide	Sterol	Pigment in mycelia	Pigment in soln.	Total pigment	Unused glucose	Fat coeff.
	g./l.		*g.*	*Per cent*	*Per cent*	*mg./l.*	*mg./l.*	*mg.*	*g./l.*	
Raulin-Thom[a]	25	5.1	5.48	4.3	0.56	13.7	38.8	53	0.79	0.97
	50	2.9	6.67	13.1	0.40	81.3	88.6	170	7.8	2.0
	100	2.9	5.89	9.2	0.30	32.5	69.2	102	51.9	1.12
Czapek-Dox[b]	25	6.6	4.66	4.0	0.73	6.2	68.4	75	0.00	0.78
	50	6.3	7.71	5.1	0.67	48.7	297	346	1.8	0.82
	100	4.5	4.44	15.4	0.56	83.2	84.6	168	37.4	1.09

[a] Initial pH 3.60.
[b] Initial pH 4.2.

altered metabolism, with the possible production of organic acids. The maximum total lipide production is accompanied by maximum pigment formation in the mycelium, and also by the maximum fat coefficient, namely, 5% glucose (Raulin-Thom). However, on the Czapek-Dox medium, these maxima are attained at the 10% level of glucose.

A detailed study of the growth of FsD on 2.5, 5, and 10% glucose solutions to establish the relationship between fat, sterol, and pigment production, and carbohydrate conversion factor was also carried out. The FsD in these experiments was grown on the basic Czapek-Dox medium. The results of these experiments are presented in Table VII and Fig. 2.

TABLE VII

*Interrelationship Between Fat, Sterol and Pigment Production
in Fusarium solani D₂ Purple (FsD)*[a]

Glucose in medium	Time	pH	Average mat weight	Sterol	Pigment in my-celia	Pigment in soln.	Total pigment	Nitrogen	Unused glucose	Fat coeff.
g./l.	*Weeks*		*g.*	*Per cent*	*mg./l.*	*mg./l.*	*mg.*	*Per cent*	*g./l.*	
	1	5.4	3.41	0.68	3	52	55	6.95	9.3	0.91
	2	5.9	4.55	0.56	4	50	54	6.07	0.7	0.66
25	3	6.6	4.66	0.73	6	68	74	6.07	0.0	0.78
	4	6.8	4.56	0.68	7	67	74	5.45	0.0	0.68
	5	7.1	3.71	0.61	6	63	69	5.58	0.0	0.49
	1	4.5	2.17	0.61	11	28	39	7.13	26.3	0.51
	2	5.2	5.06	0.59	37	280	317	6.49	9.3	0.59
50	3	6.3	7.71	0.67	49	297	346	6.34	1.8	0.82
	4	6.7	6.61	0.68	46	284	330	5.79	0.2	0.60
	5	7.0	5.94	0.71	37	276	313	5.60	0.2	0.61
	1	4.3	0.89	0.58	0	5	5	7.08	81.3	0.23
	2	4.4	2.48	0.70	1	9	10	5.78	62.9	0.51
100	3	4.5	4.44	0.56	83	85	168	5.74	37.4	1.09
	4	4.6	5.66	0.61	82	123	205	5.56	9.7	1.00
	5	5.2	5.42	0.58	104	196	300	3.17	5.3	1.93

[a] The FsD was grown on a Czapek-Dox medium, initial pH 4.2.

COMMENTS

Sterol formation in FsD reaches its maximum after the first week and thereafter remains practically constant, within limits of experimental errors. This seems to indicate that the sterol serves its purpose during the first week of growth.

On the 2.5 and 5% glucose solutions, the amount of fat produced, like the sterol, reaches its maxmum value during the first week of growth, and thereafter remains fairly constant. On the 10% glucose, the

fat increases instead of remaining constant, reaching its maximum value of 33.8% at the end of the fifth week.

On the 2.5 and 5% glucose solutions, the total pigment production is the same after the first week; *e.g.*, there is no more pigment produced
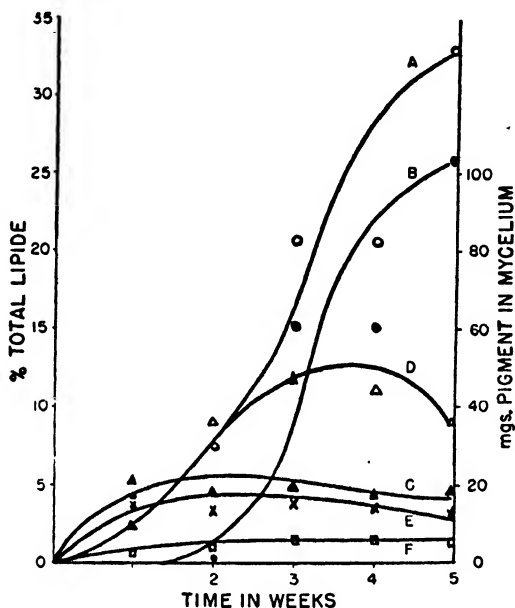
FIG. 2. Fat and pigment production in FsD mycelium when grown on Czapek-Dox medium, employing various conc. of glucose.

○A Fat production on 10% glucose.
●B Pigment production on 10% glucose.
▲C Fat production on 5% glucose.
△D Pigment production on 5% glucose.
×E Fat production on 2.5% glucose.
□F Pigment production on 2.5% glucose.

and it will be noticed that there is no more fat produced after the first week. However, on the 10% glucose solution, the pigment production, as the fat production, increases and reaches its maximum at the end of the fifth week. But the most striking relationship rests on the amount of pigment retained in the mycelium accompanied by a simultaneous occurrence of a fat maximum. For example, the greatest increment in fat production is paralleled by an increment in pigment production. Consequently, there seems to be a definite relationship between the quantity of pigment and the amount of fat formed.

The percentage of nitrogen decreases in all cases as the mold grows, and this may indicate that the process of disintegration begins after the first week.

The carbohydrate conversion factor reaches a maximum value of 1.93 at the end of the fifth week, paralelled by the maximum of pigment formed in the mycelium (104 mgs.) and by the maximum amount of fat (33.8%) on 10% glucose.

There seems to be, therefore, a relationship between carbohydrate conversion, fat formation, and pigment production. The relationship seems to involve a hydrogen transport system or an enzyme system, the carrier of which contains -SH groups, but whether the pigment affects both systems has yet to be shown. However, it seems to be obvious that the presence of the pigment is quantitatively connected with the conversion of carbohydrate to fat.

SUMMARY AND GENERAL CONCLUSIONS

1. When a pigment, *e.g.*, FsD pigment, is added to a growing non-pigment producer, FlB, there is a 50% decrease in mycelial weights, and the carbohydrate conversion factor is greatly lowered, the maximum effect being on 2.5% glucose.

2. When Flyco produces a pigment, *e.g.*, on Raulin-Thom medium, the fat and pigment increases with increasing concentration of glucose, maximum fat formation coinciding with maximum pigment production. When no pigment is produced, *e.g.*, on Czapek-Dox medium, the fat decreases with increasing concentration of glucose, the organism following a carbohydrate-fat metabolism similar to FlB when grown on either Raulin-Thom or Czapek-Dox.

3. When FsD is grown on Raulin-Thom medium, the maximum total lipide formation and the maximum fat coefficient are accompanied by maximum pigment production in the mycelium, these maxima being on 5% glucose.

4. When FsD is grown on Czapek-Dox medium, maximum lipide formation and the highest carbohydrate conversion factor are accompanied by maximum pigment production in the mycelium, these maxima occuring on 10% glucose. On 2.5 and 5% glucose solutions, the fat and sterol formation reach their maxima after the first week, while, on a 10% solution, there is an increase in fat and pigment production but not in sterol after the first week. Thus, there is no relationship between sterol and fat formation.

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A Comment on the Absorption Bands
of Ferricytochrome c

In the *Advances in Enzymol.* **7**, 281 (1947), I have stated that the ferricytochrome absorption band at 692 mμ, described as a new observation by Horecker and Kornberg (1) in 1946, was described by Bigwood *et al.* (2, 3) in 1934. Dr. Horecker has pointed out to me that Bigwood's bands at 675 and 640–45 mμ appeared only in alkaline solution and thus cannot be the same as those appearing in neutral solution. Renewed study of the papers of Bigwood *et al.* has convinced me that Dr. Horecker is right in this respect. This, however, does not mean that Horecker and Kornberg are justified in claiming any priority on the discovery of the ferricytochrome bands at 695 and 655 mμ in neutral solutions, since these bands were described by myself in 1940–43 (4, 5, 6). I regret having credited Bigwood *et al.* with an observation made by myself. Horecker and Kornberg in their paper quote neither Bigwood's nor my papers.

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Function of Alpha-Tocopherol in Lipoxidase Metabolism

In the paper "Crystalline Lipoxidase. II. Lipoxidase Activity," which appeared in the December 1947 issue, R. T. Holman states that "α-Tocopherol caused a 25% inhibition (of lipoxidase activity) at 4×10^{-4} M. It is likely, however, that this inhibition is due, not to a true enzyme inhibition, but to antioxidant action of these phenols." Referring later to estimates of the substrate turnover of the enzyme, he says "Inasmuch as the oxidation of linoleic acid, once initiated, can proceed *via* a chain reaction, it is impossible to say how much of this turnover is due to chain reactions and how much is due to direction action of lipoxidase."

We believe that Dr. Holman's results resolve the very difficulty mentioned. α-Tocopherol would serve the double purpose of permitting the wanted oxidation by lipoxidase to occur and repressing the unwanted side and continuing oxidation; this would indeed be one of the physiological services performed by vitamin E in tissue. As a corollary, measurements made in the presence of vitamin E should yield the true value of substrate turnover.

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    Received February 26, 1948

Book Reviews

It is not often that those interested in the field have served to them all at one time such a toothsome diet of general microbiology as this jubilee volume issued in honor of Professor Kluyver upon the occasion of his 25th aniversary in the Chair of Microbiology at the Technical University at Delft. Few men of science are accorded during their lifetime the organized homage of their peers, especially in this tangible form, and one has only to peruse the charming introductory testimony by Jan Smit on behalf of the Netherlands Society of Microbiology and the list of contributors to witness the personal and scientific esteem in which the honoree is held by scientists of all countries.

Workers in virtually every non-medical aspect of microbiology need no reminder of the impact Kluyver and his school have made on General Microbiology through this quarter century. Not all, however, may be aware that Kluyver is the direct successor to Beijerinck in Delft. It would be almost plagiarizing Smit's testimony to tell how successfully Kluyver has perpetuated that microbiological insight and spirit. Perhaps no better indication of the power he has been in the field of microbiology is that he, like Beijerinck, has had the singular distinction of having his name adjectivized in relation to certain of his fundamental contributions. Thus it is not enough that one of his major concepts be considered as an axiom—it is a "well-tested Kluyverian axiom"! [Stanier, R. Y., *J. Bact.* **54**, 339 (1947).] And how many microbiologists are privileged to claim an anaerobic sporeforming bacterial species as a namesake?

Scanning the contents of this tome at once gives a pretty good summary of the scope of modern research microbiology, from the standpoint of fields, problems, methodology, and tools (isotopes and even moving pictures!). It is impossible to treat each article in detail, but a few merit comments for various reasons. Probably most appealing to the collective reader will be those articles in which the authors, by virtue of their experience and authority, develop concepts, point out generalizations, and create a coordinating picture from what otherwise is a series of isolated and seemingly not very significant phenomena. These are the articles which really teach microbiology—they are Kluyverian in nature. Here is Winogradsky on microbiological ecology, Miss Stephenson on hydrogen transfer, Thornton and also Virtanen on *Rhizobium*, Meyerhof on cell-free fermentation, Clifton on oxidative assimilation, Soriano on *Flexobacteriales* systematics, Westerdijk on cultivation of fungi, Wassink on photosynthesis and Orla-Jensen on ensilaging.

Winogradsky points out that the behavior of an organism in pure culture is no criterion of its activity in the complex of nature—that ecological relations are too little considered. This scarcely must be emphasized to modern soil microbiologists, but many others could profitably cogitate upon it. If one grants that Kluyver and

Donker's 1926 paper on *Die Einheit in der Biochemie* is his broadcast contribution to biology, it is most appropriate (and Kluyver must derive a full measure of gratification) to see in this volume 2 papers epitomizing this concept in special applications: Stephenson on hydrogen transfer dealing mainly with heterotrophs and Wassink with photosynthesis. Indeed, the essence of Starkey's anaerobic corrosion of iron by sulfate-reducing bacteria also stems from this generalized concept. If ever sulfate-reducing bacteria are associated with any place beside the mud in the canals of Holland, it will be the laboratories in Delft, for virtually all our fundamental knowledge of the morphology and physiology of this specialized group of organisms comes from this laboratory, commencing with Beijerinck in 1895, or from workers who have studied at Delft. When Kluyver, in his 1931 monograph, proposed a mechanism for biological sulfate reduction, could he have imagined his mechanism would one day explain the anaerobic rusting of iron?

Miss Stephenson expands the Kluyverian principle, rather more well-known these days than a few years ago, that biological oxidations are hydrogen transfers and that, in addition to oxygen, numerous other acceptors can function to consummate the oxidation, and that groups of bacteria or types of metabolism are specialized according to the particular H acceptor they employ (and also the reduced end-product) ranging from CO_2 to amino acids. The reviewer is aware of no other article bringing together so interpretatively information on enzymes concerned with hydrogen: hydrogenase(s), dehydrogenases, and hydrogenlyases.

Two points in this paper are puzzling: on p. 36 is a thermodynamic anomaly, or a typographical error: the energy liberated in a 2-step reduction of HNO_2 to NH_3 by H_2 (hydrogenase) is substantially in excess of the energy liberated as expressed in a 1-step overall reaction $(50 + 185 = 235 \text{ Cals. } vs. 167 \text{ Cals.})$. On p. 45 Miss Stephenson says: "The evidence that these organisms (*Athiorhodaceae*) build up cell material solely from CO_2 reduced by organic hydrogen donators, and not by any fermentative breakdown, became overwhelming only when strains of these purple bacteria were found which were able to replace the organic hydrogen donators by molecular hydrogen." As a matter of fact, a master of bacterial photosynthesis himself, C. B. van Niel, never considered this adequate evidence at all, and did not consider the issue proved until it was shown that an organic compound could function in photosynthesis as a hydrogen donor exclusively, and not lose any carbon in the process, *viz.*, photosynthetic dehydrogenation of isopropanol to acetone. The evidence which Miss Stephenson considers acceptable in this case also should permit one to conclude that because facultatively autotropic hydrogen bacteria synthesize their cell material from CO_2 with hydrogen gas, this takes place when they are put under heterotrophic conditions, and the organic substrate is broken down entirely to CO_2, then reassimilated, without any direct assimilation of intermediary breakdown products. While this may be so, it certainly is contrary to current beliefs.

Perhaps it is fate that Barker should have just rounded out the main fermentation characteristics of *Clostridium Kluyveri* in time to have it appear in this Festschrift volume. For more than name alone will this organism be famous, for here is clear-cut evidence of the mechanism of the synthesis of fatty acids with 4, 5, 6, and 7 carbon atoms from simpler 2, 3, and 4 carbon chains—obviously a model for synthesis of all the higher fatty acids. Acetyl phosphate condensation with acetic, propionic, butyric, or valeric acids gives the 4, 5, 6 and 7 carbon fatty acid. Though alcohol is the starting

material, it is simply the raw material for acetyl phosphate, and chains are built up by compounding this C_2 fragment with even and odd number fatty acids. The classical ideas of fat synthesis from acetaldehyde condensations surely will have to give ground in the face of this basic work.

Space does not permit similar commentaries on the other papers in this volume. It should be mentioned, however, that several are regular scientific articles presenting original results: Gross and Werkman on conversion of labeled acetaldehyde, Neuberg and Roberts on saccharase, Keilin and Hartree on respiratory activity of bacterial spores, Winge and also Lodder on yeast genetics, van Niel on carotenoids of photosynthetic bacteria, Chaix, Chauvet, and Fromageot on respiration of a protozoan, Westerdijk on the problems of maintaining the internationally known fungus culture collection at Baarn. The newer fads of microbial nutrition and of antibiotics come in for their share in papers by Schopfer on inositol and *Eremothecium ashbii*, the riboflavin-producing fungus, and Waksman on the *Actinomycetes*. Methodology is well represented: Pijper on moving pictures in microbiological research, Kingma Boltes on photomicrography, Elema on estimation of copper by electro-deposition. Applied microbiology is not neglected: Orla-Jensen and collaborators describe the practice of silaging, Thaysen and Morris tell of food yeast manufacture in the British West Indies, and Broekhuizen reports on prevention of ropiness in bread making. But the most fascinating application of microbiology is engagingly narrated by G. Giesberger and covers his experiences during several years in Japanese camps for prisoners of war. One can only marvel at the ingenious applications of general microbiology under the primitive conditions prevailing in a crowded prison camp. Production of yeast for bread baking, sweet tasting hydrolyzate of rice, food yeast to combat protein and Vitamin B_1 deficiencies, distilled spirits for medicinal (and consumption) purposes, vinegar, digestible soy bean protein foods by controlled mold growth are those developed to relieve the food problem. But others, even more ingenious, were developed—$CaCO_3$ was produced from lime water with CO_2 generated by a microbiological process. The precipitated chalk was employed as an astringent in diarrhea cases and as a raw material for toothpaste. Giesberger assures the reader that had not liberation arrived, he would have had urea bacteria working for him to produce ammonia from urine as a lye in the manufacture of soap. Truly here is the culmination of the principles of general microbiology in the true Delft tradition, and Giesberger often must have reflected how good his fortune was to have acquired as a student the background for this knowhow from his microbiological mentor—Kluyver himself.

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The Fungi. By FREDERICK A. WOLF, Department of Botany, Duke University, and FREDERICK T. WOLF, Department of Botany, Vanderbilt University. John Wiley & Sons, Inc., New York, 1947, Chapman and Hall, Ltd., London. Vol. 1, x + 438 pp., 153 figs. Price $6.00. Vol. 2, xii + 538 pp., 81 figs. Price $6.50.

Since these two volumes present a departure from the usual approach to mycology, the breakdown into large sections is given for each volume together with the number of pages allotted to each heading. The books are appropriately dedicated to the memory of de Schweinitz, "the father of American mycology," for the double reason that he was the most prominent early American student of fungi, and that he worked in the Carolinas as does the senior author. In the chapter upon "Founding," pictures of Spegazzini, Atkinson, Farlow, and Harper are added.

Contents, volume I:

1.* The Founding of Mycology, 13 pages; 2. Isolation and Cultivation of Fungi, 15 pages; 3. Classification and Taxonomy of Fungi, 29 pages; 4. The Myxomycetes, 17 pages; 5. The Phycomycetes, 78 pages; 6. The Ascomycetes, 139 pages; 7. The Basidiomycetes, 100 pages; 7. The Deuteromycetes (Fungi imperfecti), 20 pages; Author index, 7 pages; Subject index, 26 pages.

Volume II:

1. Nutrition of Fungi, 36 pages; 2. Enzymes and Enzymic Activities, 16 pages; 3. Respiration, 16 pages; 4. Biochemistry, 27 pages; 5. Effects of Temperature, 27 pages; 6. Effects of Radiation, 28 pages; 7. Effects of Reaction of Substrate, 15 pages; 8. Spore Dissemination, 44 pages; 9. Germination of Spores, 26 pages; 10. Host Penetration, 21 pages; 11. Physiologic Specialization and variation, 22 pages; 12. Associative Effects, 18 pages; 13. Mycorrhizae and Mycotrophy, 20 pages; 14. Genetics, 22 pages; 15. Poisonous and Edible Fungi, 25 pages; 16. Medical Mycology, 31 pages; 17. Geographical Distribution, 21 pages; 18. Mycology in Relation to Plant Pathology, 13 pages; 19. Soil Fungi, 13 pages; 20. Fungus-Insect Interrelationships, 16 pages; 21. Marine Fungi, 16 pages; 22. Fossil Fungi, 17 pages; Author index, 11 pages; Subject index, 37 pages.*

The chapter headings in the table of contents are well broken down and each subhead is indexed to the page. The whole content of both volumes is thus made readily accessible.

Indexes are good: both author and subject indexes make all information quickly available. There is no combined index for the two·volumes, which are assumed to diverge so sharply in content that a general index is not needed, however useful when the volumes are used for reference purposes.

Glossary:

Where so many technical terms are used, some of them coined for this book, and others used in restricted senses, a glossary would be better than scattered paragraphs defining special terms.

The change of attitude appears first in the introduction, as Ch. 2, of Isolation and Cultivation of Fungi. The practices described are mainly those used in plant pathological laboratories and for special groups. The user is assumed to have at hand other manuals for general culture practices. The remainder of Vol. I covers the groups of fungi. The only concession to the beginner who needs some clue to the identification by group, of an organism in hand, is a Key (Vol. I, pp. 34–39). The groups of fungi are presented without detailed keys within the larger units, or sectional breakdowns. Apparently, they expect the student, if outside some classroom, to find his clue to group nomenclature by looking at the figures. In the classroom, the student is furnished the necessary illustrative material. Scrutiny of many of these chapters, each supported by a bibliography, shows that much of the presentation is based upon recent research literature. The descriptive material and the comments of the authors testify to their wide acquaintance with fungi, especially in those groups which have been recently brought into cultivation.

The two volumes present a tremendous amount of work in the laboratory, in the field, and in the library. One may assume that the whole scheme of presentation has been tested fully in the classroom, by both authors.

* Chapter titles are preceded by chapter number.

Volume II brings together a very great amount of biochemical and physiological information from research sources, correctly named and cited for each chapter. Such a coordination of information from many sources cannot fail to be very useful to every student of fungi. The analysis, topic by topic, is very complete and brings together the information from many phases of physiology, ecology and biochemistry. The paragraphs labeled *Implications* at the end of many of these chapters presents the interpretations of the authors in very useful form.

It is too bad to find fault with a book covering so many phases of this subject, but the reviewer turned to it as an optimist hopeful that he might find the last word, or at least, find the latest word. Some things are a distinct disappointment. Outstanding is the need of adequate collaboration. Nowhere is this more evident then in the chapter on soil fungi, in which the work of the U. S. Department of Agriculture was practically ignored. The reviewer is keeping to his own territory—he spent 15 years on the material in this chapter. The authors failed to recognize that soil microbiology is loaded with lists of organisms obtained by standard isolation methods, with their possible activities established *in vitro* but without demonstration in the field. Much of this material, as found here, requires an entire change in viewpoint for a better field analysis of the elements of the soil population as a basis for sound interpretation.

One who has put 40 years into the study of molds in their relation to agriculture and industrial problems, and was driven in self defense to become a monographer, is just a bit irritated by the authors' pronouncement that "the taxonomic approach" has been "deëmphasized." The reader is informed that Latin binomials, as used here, are taken directly from research papers cited (no matter how crude the mistake in nomenclature). He is gravely informed that he can consult Saccardo as to names if he does not like what he finds here. There is no recognition that many laboratories do not have access to Saccardo, and very many workers are without the extensive taxonomic training to use the "Sylloge" if they had it. We find, for example, biochemical reactions cited for *Eidamia catenulata* and for *E. viridescens* without recognition that the molds so masquerading belonged to different genera neither of which is *Eidamia*. Again, there are citations of the activities of *Penicillium glaucum* with no warning that that binomial as found in the physiological and biochemical literature is rarely more than a synonym for *green mold*. If we could safely assume that the folks who use Latin binomials, or even some of those who perpetrate them, had the real "taxonomic approach," such deëmphasis might be excusable.

The illustrations are "mostly adapted from those of others." What does adapted mean? The discriminating reviewer turns to his own special field for information. Fig. 54 (Vol. I, p. 158) A, B and C, purports to represent *Penicillium roqueforti* whose conidia are globose and smooth under the microscope, but elliptical and rough in this figure. The sterigmatic cells (phialides) as figured would move the species to a different section of the genus. Whoever *adapted* the figures failed to recognize the significance of cell shapes and markings in the conidial apparatus of the molds. No attempt is made here to list errors in dates and in details of statement. What have been cited leave a doubt in one's mind about the propriety of adapting figures unless care is taken to ensure the correctness of the concept to be presented by the figure as finally presented.

The literature cited in each chapter is given at the end of the chapter, alphabetically by authors. Perhaps the placement is useful to the beginner but it is wasteful of print-

ing space because of necessary repetition and is burdensome to the experienced worker who wishes to reach the works of particular men quickly. Where more than one investigator bears the same surname it would help to introduce the initial where reference is made in the text.

Two pages (Vol. II, 86–88) devoted to "Other Metabolic Products," and pp. 280–285 covering autagonism in mixed cultures, are all the space allowed for the discussion of antibiosis in all its forms. Thus, the greatest mycological accomplishment of the age —the development of penicillin—is practically ignored. The group of workers investigating the production of penicillin has furnished a large amount of information about culture, culture media, mold physiology, induced variation, strain variation, and morphologic changes, and has coordinated these numerous findings with biochemical studies. The user of such a book as this is entitled at least to a summary of this work, which is fully as good mycology as studies of *Basidiobolus ranarum* or even *Pilobolus*.

Any criticisms, such as are offered here, are inevitable when one or two persons of the same academic background write a book covering a field in which many diverse groups are furnishing basic information. No one person can assimilate such a mass of material as this without occasional loss of perspective, which is not always corrected by reference to authorities selected as critical by workers without personal experience in those particular fields. Nevertheless, these books will take valued space upon my shelf of reference because of the mass of indexed material which I find in them.

CHARLES THOM, Port Jefferson, L. I., N. Y.

Biochemistry of Cancer. By JESSE P. GREENSTEIN, Head Biochemist, National Cancer Institute, National Institute of Health, Bethesda, Maryland. Academic Press, Inc., New York, N. Y., 1947. viii + 389 pp. Price $7.80.

The special attraction of this book is enhanced by the fact that it does not list uncritically the numerous statements contributed to the cancer problem by more or less competent observers, as far as they can be biochemically comprehended. It is meant less for clinicians and more for biologists and chemists desirous of enriching or enlarging their knowledge of this difficult field. These readers will set a particularly high value on such chapters as the classification of animal tumors, experimental induction of tumors, plant tumors, the influence of extrinsic and intrinsic factors, the chemistry of tumors in its stricter sense, and the chemistry of tumor-bearing hosts, but above all, the parts dealing with the formation of cancer by polycyclic hydrocarbons, the enzyme systems in pathological cells, and much else of interest. Most instructive is the chapter on the role of proteolytic enzymes and dehydropeptidases, in whose investigation Greenstein himself excelled, but Greenstein's own universally known research does not appear in the book; in vain does the reader look for his name in the general author index, so that one might easily overlook his share in the development.

In a new edition, a somewhat fuller treatment of the mineral metabolism of tumors would be desirable; the same applies to the chapter "Chemotherapy." The reasons why some chemotherapeutic results observed on transplanted tumors did not gain increased importance might be mentioned. This may be partly due to the special conditions of vascularization.

On the whole, the book deserves approbation and wide circulation.

C. NEUBERG, New York, N. Y.

The Effect of Insulin on the Enzyme Systems Responsible for the Glycogenic and Glycogenolytic Cycles of the Liver [1]

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INTRODUCTION

The most characteristic disturbance in insulin insufficiency in man or animals, and the one most rapidly returned to the normal state subsequent to the injection of insulin, occurs in the mechanisms involved in the formation, storage and breakdown of liver glycogen. This and other facts gained from a study of metabolic phenomena make it appear probable that insulin must exert a profound effect somewhere in the liver cycle represented by the overall equation:

$$\text{Glucose} \leftrightarrows \text{Glycogen}$$
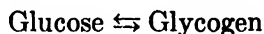
The various steps represented in this transformation have been clarified largely as a result of the classical studies of Cori and his collaborators. These steps and the enzymes known to participate as well as the references to the original work are appended below:
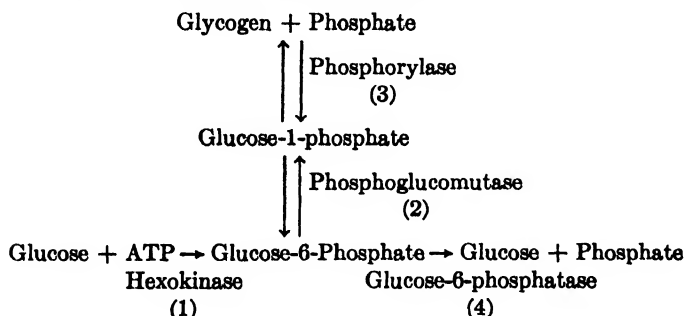
Glycogen + Phosphate

⇅ Phosphorylase
(3)

Glucose-1-phosphate

⇅ Phosphoglucomutase
(2)

Glucose + ATP → Glucose-6-Phosphate → Glucose + Phosphate
Hexokinase   Glucose-6-phosphatase
(1)   (4)

In addition, at least one more known enzyme is probably concerned with this phase of the carbohydrate cycle, namely:

$$\text{Phosphorylase a} \rightarrow \text{Phosphorylase b}$$
$$\text{PR}$$
$$(5)$$
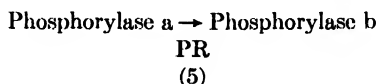
Obviously, the effect of insulin may be exerted directly on the enzyme systems concerned in these reactions. On the other hand, the action of insulin may be directed, particularly in the intact cell, on mechanisms which themselves mobilize or regulate the activity of the enzyme systems enumerated above.

A number of reports have appeared concerning the effect of insulin on certain of these systems. Insulin has been reported to have no effect on the phosphorylase of muscle (6). The effect (7) and lack of effect (8) of insulin on the phosphoglucomutase system of muscle have been described, but no data appear to be available for liver phosphoglucomutase. Colowick, Cori and Slein (9) have described the decreased activity of hexokinase extracts from muscles and livers of alloxanized rats and the increase of this activity after the addition of insulin. This effect was attributed, not to a direct action of insulin on the hexokinase system *per se*, but rather to the counter-inhibition of an inhibitory factor presumably originating from the pituitary. Although we entertain no doubts concerning the validity of these observations, we (10), as well as others (11), have been unable to demonstrate either the decreased activity of extracts of diabetic tissues or the stimulating effects of insulin *in the absence of added large amounts of pituitary or adrenocortical factors*. Up until now, no direct observations have been reported in regard to the effect of insulin on glucose-6-phosphatase. Recently, however, we were able (4) to characterize this system, and to describe a method for the determination of its activity.

Due to the lack of data on certain of these systems and the conflicting information on others, and the fact that they have not been studied at one time or in the same laboratory, it appeared worth while to reinvestigate this problem. Accordingly, we set out to measure the effects of insulin on the various systems enumerated above.

METHODS

If the action of insulin occurs directly on an enzyme system, it should be possible to detect the effect of insulin on cell-free extracts containing the enzyme system in question. If, on the other hand, the activity of insulin is directed primarily toward the mobilization or regulation of the activity of these systems, such an action might be demonstrable in the intact cell but not in a cell-free extract in which

the protoplasmic structure has been destroyed. Accordingly, in an effort to determine the effect of insulin on the enzyme systems themselves, use was made of cell-free extracts. These were prepared from the tissues of either the rat or rabbit. In many instances, in order to detect any action of insulin on extracts obtained from diabetic animals, such extracts were obtained from the tissues of alloxanized animals. Blood sugar levels, determined by the Nelson procedure (12), were performed on samples taken at the time of the sacrifice of such animals and only those that displayed a marked hyperglycemia were considered as diabetic.

The liver phosphorylase preparations were obtained by grinding the thoroughly perfused livers of unfasted rats with 2 volumes of ice cold water, centrifuging the mixture at a high speed, and filtering the supernatant extract through fine gauze. These extracts were then adjusted to pH 6.7 and used immediately. PR was prepared from rat liver according to the method described by Cori and Cori (13) for muscle PR. The liver extracts were adjusted to pH 6.2, and dialyzed against running water for 3 hours. The dialyzed extract was adjusted to pH 5.8 and the resulting precipitate collected and washed with distilled water and centrifuged. Unmeasured small volumes of water were added to this precipitate and the mixture adjusted to pH 8.5, during which time some of the precipitate appeared to dissolve. After being stored over night in the deep freeze, the frozen mixture was permitted to thaw and the insoluble residue removed. The supernatant fluid was adjusted to pH 6.7 or 6.2 and utilized as the source of PR with no attempt at further purification.

The extracts containing phosphoglucomutase and glucose-6-phosphatase activity were obtained from rat liver by methods previously described (4). Hexokinase-containing extracts were obtained from liver and muscle by the method described by Colowick, Cori and Slein (9).

The quantitative estimation of phosphorylase activity in crude liver extracts presents a difficult problem. The conventional method for the determination of such activity is based upon estimation of the rate at which glucose-1-phosphate is converted to glycogen and inorganic phosphate (14). In crude muscle extracts such a procedure has been applied with success due to the absence in such extracts of a hexosemonophosphatase. The very active phosphoglucomutase and glucose-6-phosphatase systems prevent the application of this procedure to crude liver extracts, since these latter systems act to convert the substrate to glucose and inorganic phosphate rather than to glycogen.

Inasmuch as the phosphorylase reaction is reversible, it was considered advantageous to attempt to utilize a method based upon the rate of breakdown of glycogen by crude liver extracts. Since extracts

prepared from well-perfused livers contain little diastase, the phosphorolytic disruption of glycogen by such extracts must be attributed largely to the activity of the contained phosphorylase.

Certain theoretical objections prevent the use of such a procedure for the quantitative determination of phosphorylase activity. The rate of breakdown of glycogen by such extracts can obviously be influenced not only by the activity of the phosphorylase system but also through the intervention of the phosphoglucomutase and glucose-6-phosphatase systems, both of which tend to accelerate the rate of breakdown of glycogen as a result of their continued action in removing the product of phosphorylase activity (glucose-1-phosphate). Accordingly, although the rate of breakdown of glycogen in such extracts is a measure of their glycogenolytic activity, it does not describe accurately the activity of the contained phosphorylase.

On the other hand, it will be demonstrated that the addition of insulin has no effect on the activity of either phosphoglucomutase or glucose-6-phosphatase. Therefore, in spite of the fact that the rate of breakdown of glycogen by extracts of perfused liver does represent a combination of phosphorylase, phosphoglucomutase and glucose-6-phosphatase activity, if the addition of insulin produced any influence on the activity of the phosphorylase factor in these combined systems, such an effect should be evidenced by some change in the rate of glycogenolysis, since, in both the presence and absence of insulin, the activities of the phosphoglucomutase and glucose-6-phosphatase factors would remain unchanged.

In accordance with such considerations, we have attempted to estimate, not phosphorylase activity *per se*, but rather the effect of insulin on this activity by comparison of the rates of glycogenolysis in the presence and absence of insulin. For this purpose, 1% glycogen was added to the reaction mixture to augment that already contained in the extracts and the glycogen content of the reaction mixture before and after incubation was determined by the method of Good *et al.* (15). The activity of crystalline muscle phosphorylase was determined by the procedure described by Cori, Cori and Green (14).

Various attempts to determine the effect of liver PR on the activity of the phosphorylase obtained from liver led to erratic results. The activity of liver PR was, therefore, estimated by measuring its effect, according to the method outlined by Cori and Cori (15), on a recrystallized preparation of phosphorylase 'a' obtained from rabbit muscle (16). Phosphoglucomutase and glucose-6-phosphatase activities were assayed by determination of the changes in the P_0 and P_7 values of the reaction mixture containing glucose-1-phosphate under conditions previously described (4). The validity of this method depended upon the demonstration that the inorganic phos-

phate liberated from this substrate was derived solely from the activity of glucose-6-phosphatase on Robison ester, and therefore, that the rate of formation of inorganic phosphate from Cori ester was a measure of the activity of the glucose-6-phosphatase. Similarly we demonstrated that the phosphoglucomutase activity could be determined through a study of the rates of change of both P_7 and P_0 values since the amount of conversion of glucose-1-phosphate to glucose-6-phosphate in such a reaction mixture could be calculated from the expression: glucose-6-phosphate formed $= \Delta P_0 - \Delta P_7$.

The insulin[2] utilized in this study, unless otherwise noted, consisted of a highly purified amorphous sample containing approximately 22 units/mg. The crystalline trypsin and soy-bean trypsin inhibitor specimens were obtained through the kindness of Dr. M. Kunitz.

RESULTS

The several preparations of the same extracts obtained as described above failed to exhibit a uniform activity. Thus, preparations of liver phosphorylase obtained from different lots of animals often displayed markedly different activities. These differences could in no way be correlated with the fact that the animals had or had not been alloxanized. Accordingly, differences in the activity of preparations from diabetic and normal animals could not be attributed to the presence or absence of diabetes. In the tabulation of results, such differences in activity may often be noted and no implication should be placed upon these differences. On the other hand, in the discussion to follow, it is desired to focus attention on the effect of the addition of insulin on the activity of any one particular preparation of extract.

A. Phosphorylase

The addition of insulin had no appreciable effect on the glycogenolytic activity of fresh crude extracts from perfused livers or on the activity of a recrystallized specimen of phosphorylase 'a' from rabbit muscle (Table I), thus confirming the findings of Cori, Colowick and Cori (6).

B. 'PR'

As noted above we were not able to obtain reliable results concerning the effect of liver PR on the phosphorylase contained in crude liver extracts. In Table II, there is demonstrated the lack of any real effect of insulin on the rate of destruction of crystalline phosphorylase 'a' from muscle by liver PR.

[2] We are indebted to the Eli Lilly Co. for a generous supply of this insulin.

TABLE I

Effect of Insulin on Glycogenolytic[a] Activity of Crude Liver Extract
and Activity of Crystalline Muscle Phosphorylase[b]

Source of phosphorylase system	Insulin γ	Glycogen mg./ml.			Inorganic phosphate γ/ml.		
		Before incubation	After incubation	Amount broken down	Before incubation	After incubation	Amount formed
Diabetic rat liver	0	11.4	5.7	5.7			
	40	11.4	5.7	5.7			
Normal rat liver	0	14.6	5.1	9.5			
	40	14.6	5.2	9.4			
Crystalline rabbit muscle phosphorylase 'a'	0				3	198	195
	40				3	198	195

[a] Glycogenolytic activity expressed in terms of breakdown of glycogen during 20 minutes' incubation at 30°C. and pH 6.7. The final reaction mixture of 2 ml. contained 1% added glycogen, $M/100$ phosphate, 0.015 M cysteine, 10^{-3} M adenylic acid, and 0.6 ml. of liver extract.

[b] Phosphorylase activity determined as described in legend to Table III without the preliminary incubation at 25°C.

TABLE II

Effect of Insulin on Destruction of Crystalline Muscle
Phosphorylase a by Liver PR

Added components		Phosphorylase activity[a]			
Source of PR	Insulin γ	P_0 γ/ml.			Per cent inhibition by PR[b]
		Before incubation	After incubation	Formed during incubation	
None added	—	5	184	179	0
	40	6	190	184	0
Normal liver	—	6	25	19	89.5
	40	7	23	16	91.1
Diabetic liver	—	6	18	12	93.3
	40	6	20	14	92.2

[a] Determined as described in legend to Table III.
[b] Expressed as inhibition of inorganic phosphate liberation.

In connection with PR, the similarity of its action to that of a proteolytic enzyme was noted by Cori and Cori (5), who described certain points of resemblance between the destruction of phosphorylase 'a' by both PR and trypsin. It therefore appeared to be of some interest to investigate the effect of insulin on the inactivation of phosphorylase 'a' by trypsin. Furthermore, since PR and trypsin resemble one another to some degree in their action on phosphorylase 'a', and since antitrypsins are known to be effective in inhibiting proteolysis by tryptic enzymes, we attempted to determine the effect of a crystalline trypsin-inhibitor obtained from soy bean and the effect of the antitryptic agent in human serum on the action of both trypsin and PR on phosphorylase 'a' from muscle. These results are shown in Table III,

TABLE III

Effect of Insulin and Trypsin Inhibitors on the Inactivation of Crystalline Muscle Phosphorylase by Muscle PR and Trypsin

Added components					Phosphorylase activity[a]			
					P_0 γ/ml.			Per cent inhibition of phosphorylase[b]
PR ml.	Insulin γ	Trypsin γ	Soy bean inhibitor γ	Human serum ml.	Before incubation	After incubation	Formed incubation during	
—	—	—	—	—	5	167	162	0
0.1	—	—	—	—	5	13	8	95.1
0.1	40	—	—	—	7	15	8	95.1
0.1	—	—	40	—	5	15	10	93.8
0.1	—	—	—	0.2	8	17	9	94.5
—	—	—	—	—	5	199	194	0
—	—	60	—	—	5	14	9	95.4
—	0.2	60	—	—	5	13	8	95.8
—	—	60	40	—	4	209	205	0
—	—	60	—	0.2	5	215	210	0

[a] Phosphorylase activity indicated by amount of inorganic phosphate liberated from glucose-1-phosphate during a 10 minute incubation with the reaction mixture described by (13) subsequent to a preliminary 30 minute incubation at 25°C. with the components indicated in the table. During the preliminary incubation, the mixture consisted of 0.6 ml. of a 1/200 dilution of a suspension of phosphorylase crystals, the indicated additions and 0.03 M cysteine to a total volume of 1.0 ml. 1 ml. of the substrate was then added for the determination of phosphorylase activity. For the trypsin series, reactions were performed at pH 6.2, for the PR series at pH 6.7.

[b] Expressed as per cent inhibition of inorganic phosphate liberation.

which illustrates that both of the trypsin inhibitors effectively suppressed the inactivation of phosphorylase 'a' by trypsin, but had no effect on PR activity. Furthermore, insulin failed to influence the course of tryptic inactivation of phosphorylase 'a'.

C. Phosphoglucomutase and Glucose-6-Phosphatase

The activity of these systems, obtained from both alloxanized and normal animals, and the effect of insulin thereon were determined by following the course of reaction with added glucose-1-phosphate as the substrate (4). In general, the average glucose-6-phosphatase activity of the extracts obtained from the diabetic animals appeared to be somewhat higher than in the case of extracts obtained from normal animals. However, overlapping of the results obtained from extracts from the two groups occurred to such an extent that it appears unwarranted at present to draw any conclusions concerning these observations.

As is seen in Table IV, the addition of insulin failed to affect the

TABLE IV

*Effect of Insulin on Phosphoglucomutase and Glucose-6-Phosphatase
Systems in Crude Liver Extracts*

Anaerobic incubation under conditions described in (5). Initial concentration of glucose-1-phosphate 0.008 M. 0.5 ml. of liver extract in a total volume of 2.4 ml.

Source of liver extract	Insulin γ	Data given γ/ml.	Duration of incubation in minutes				
			0	5	10	20	30
Normal rat	None	P_0	22	57	73	106	131
		P_7	264	139	83	115	141
		$\Delta P_0 - \Delta P_7$		160	232	233	232
	200	P_0	22	55	74	109	129
		P_7	258	135	81	119	142
		$\Delta P_0 - \Delta P_7$		156	229	236	233
Diabetic rat	None	P_0	24	47	79	119	152
		P_7	262	172	96	127	159
		$\Delta P_0 - \Delta P_7$		113	221	230	231
	200	P_0	24	50	77	115	155
		P_7	263	172	98	127	163
		$\Delta P_0 - \Delta P_7$		117	218	227	231

activities of either the phosphoglucomutase, or the glucose-6-phosphatase systems from either diabetic or normal animals. These findings demonstrate that the conclusions of Cori *et al.* (8) in regard to the lack of effect of insulin on the phosphoglucomutase system of muscle can be extended to the same system in liver. We were also unable to confirm the observations of Gill and Lehmann (7) that insulin depresses the phosphoglucomutase of muscle. It is apparent that the glucose-6-phosphatase mechanism is not influenced by the addition of insulin.

In all of the results detailed above, similar findings were observed when various quantities of insulin were added to the reaction mixtures. The examples cited in the different tables are representative of all of our observations, which, for the sake of brevity, are not presented in detail.

Our failure to confirm the findings of Colowick, Cori and Slein (9) in regard to a decreased hexokinase activity of extracts obtained from alloxanized rats, and the increase of this activity by the addition of insulin, has already been reported (10) and it did not appear desirable to repeat such results in this present paper nor is it pertinent to detail our confirmation of their observation that insulin can exert an *in vitro* effect. It will be sufficient to state that our results with liver hexokinase differed in no way from the previously reported data (10) concerning the lack of effect of the administration of alloxan and the addition of insulin on the activity of muscle hexokinase-containing extracts.

DISCUSSION

Price, Cori and Colowick (17) state that insulin has no effect on hexokinase extracts from normal animals but exerts its activity through counterinhibition of the excess of pituitary or adrenal factors in the diabetic state. Presumably, therefore, in their opinion, insulin has no effect on the isolated hexokinase system, and this conclusion is in complete agreement with our own observations.

In this study, we have failed to observe an appreicable effect of insulin on the phosphorylase, phosphoglucomutase and glucose-6-phosphatase systems in liver extracts. Furthermore, insulin was found to have no effect on the activity of liver PR as judged by its rate of destruction of muscle phosphorylase 'a'. Our findings, therefore, confirm those of Cori and his colleagues (6, 8) in regard to muscle phosphorylase and phosphoglucomutase.

The nature of the phosphorylase system as it exists in the intact liver cell has not been clarified. Efforts to obtain phosphorylase 'a' from liver have been unsuccessful, presumably due to the rapid rate of its conversion to phosphorylase 'b' by the highly active liver PR (5).

Apparently, in extracts such as those utilized in this study, the phosphorylase is present in the 'b' form. The possibility that liver phosphorylase may also exist in the intact liver cell as phosphorylase 'a' might be inferred from analogy to muscle phosphorylase and to the presence in liver of an active PR which, so far as is known, acts specifically on phosphorylase 'a'. The fact that glucose-1-phosphate is converted to an amylose in the presence of muscle phosphorylase, and to glycogen in the presence of the phosphorylase from liver, cannot be accepted as proof of the lack of identity of the two systems. The apparent dissimilarity of their activity can be explained through the assumption that liver phosphorylase also converts glucose-1-phosphate to an amylose, which latter is reconverted to glycogen through the simultaneous activity of a second enzyme system apparently not found in extracts of muscle (18).

It is accordingly difficult to exclude the possibility that phosphorylase 'a' plays an important role in the glycogenolytic cycle in the intact liver. Since we have been unable to examine the effect of insulin on such a system, the possibility exists that, in the intact liver cell, insulin exerts its characteristic effect by means of some influence on this enzyme. If, however, phosphorylase 'a' from liver does resemble the phosphorylase 'a' from muscle, it would appear improbable that insulin could exert any influence on such a system. Furthermore, the lack of an effect of insulin on the phosphorylase in liver extracts and the similarity between the action of phosphorylase 'a' and phosphorylase 'b' in the presence of adenylic acid, make it appear improbable that insulin can directly affect the activity of any phosphorylase system. At any rate, it may be stated, as a result of these studies, that insulin has no effect on the activity of those enzymes in liver *extracts* which participate in the glycogenolytic and glycogenic cycles.

In view of the unanimity of opinion concerning the lack of effect of insulin on the hexokinase enzyme *per se*, this present study indicates a complete absence of any direct effect of insulin on any of the enzyme systems known to be concerned with the glucose $\rightleftarrows$ glycogen cycle of the liver. In passing, it may be pertinent to note that results similar to those obtained from rat liver extracts have also been observed with extracts prepared from the liver of the rabbit, Nevertheless, in the intact organ, all evidence leads to the conclusion that insulin does exert its characteristic physiological action somewhere in this cycle. Such a consideration, together with the experimental evidence offered

here (both new and confirmatory) makes it appear not unlikely that insulin acts in the intact cell through a mechanism other than a direct one on the enzymes immediately concerned with the glucose $\rightleftarrows$ glycogen cycle. In view of the well known objections which militate against the acceptance of the *in vitro* hexokinase effect as an exclusive explanation of the physiological action of insulin, it is necessary to search elsewhere for this mechanism whereby the hormone plays its role in the animal economy.

Sutherland and Cori (19) have recently reported another effect of insulin on the glycogenolytic cycle in the liver. This effect was manifested in liver slices but not in extracts. Such findings agree well with the conclusions of this present study and emphasize anew the fact that insulin has no direct action on the systems concerned in the regulation of the blood sugar level. Whether insulin acts in the intact cell by mobilizing or immobilizing substrate or enzymes is a problem for future solution.

SUMMARY

Cell-free extracts obtained from the livers of normal and alloxanized rats were utilized for the demonstration of the enzyme systems known to be concerned with the glucose $\rightleftarrows$ glycogen cycle of this organ. The systems studied included the phosphoglucomutase, phosphorylase, PR and glucose-6-phosphatase mechanisms.

The addition of insulin had no effect on the activity of any of these systems obtained from either normal or diabetic animals. In view of earlier demonstrations of the lack of effect of this hormone on the isolated hexokinase system, it is necessary to conclude that insulin must exert its influence on the glycogenic and glycogenolytic cycle of the liver by some mechanism other than a direct effect on the enzyme systems presumed to be responsible for the entire series of transformations involved in these cycles.

Insulin has no effect on the inactivation of muscle phosphorylase by trypsin. Normal human serum and soy bean trypsin-inhibitor, both of which inhibit the tryptic inactivation of phosphorylase, fail to affect the inactivation of phosphorylase by PR.

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Biochemical Mutants Affecting the Growth and Light Production in Luminous Bacteria

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INTRODUCTION

Ideas concerning the mechanism of luminescence and the relation of this system to the main respiratory pathway have come primarily from the chemical studies on extracts from the crustacean, *Cypridina hilgendorfii*, and from inhibitor studies on luminous bacteria (see 7). Since it has not been possible to extract the light system from luminous bacteria little is known concerning the chemistry of the substrate, luciferin, or the enzyme, luciferase, in these forms. Indirect evidence indicates, however, that the bacterial luciferin is a naphthoquinone derivative and that the light reaction is closely associated with the cytochrome system (9, 12, 16).

Very few studies have been concerned with the nutritional requirements for growth and light production in these organisms, although Doudoroff (3) has made the interesting observation that some, but not all, naturally occurring dim strains of luminous bacteria can be made to luminesce brightly if grown in the presence of riboflavin. Giese (5) has investigated some of the differences between dim and bright variants of *Achromobacter fischeri*. Studies similar to those reported by Doudoroff and Giese have not been made. Several studies have been made on the effects of various salts on luminescence, but in most cases no distinction between the effects on growth and light production could be made (see 7). It is possible to obtain dark cultures of luminous bacteria by growing them in the absence of oxygen or in the presence of certain chemicals; however, most of these cultures return to normal light production when subsequently transferred to a normal medium.

Since Beadle and Tatum (1, 2) have clearly shown that genetic changes in *Neurospora* induced by X-rays or ultraviolet radiation affect specific biochemical steps, and

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since subsequent studies by others (11, 15, etc.) have demonstrated that biochemical variants can be obtained in bacteria, it appeared likely that such an approach to the study of growth and luminescence would be profitable.

The present study is concerned with the growth and luminescent characteristics of biochemical mutants in luminous bacteria. With the hope of obtaining mutant types which would give us more information concerning the chemical components associated with the light producing system, we have searched for 3 particular types: (1) those strains which fail to grow on the minimal medium but grow and luminesce normally when the necessary growth factor is added to the minimal medium; (2) those which grow normally on the minimal but luminesce only on the complete medium, indicating a specific block in the light system; and (3) those which fail to grow on the minimal and subsequently show a competition between light and growth systems for the added components, indicating a block in a reaction common to both luminescence and growth.

MATERIAL AND METHODS

Doudoroff (4) described a synthetic basal medium for the growth of various species of luminous bacteria consisting of: $M/30$ Sörenson KH_2PO_4-Na_2HPO_4 phosphate buffer at pH 7.0; 3% NaCl; 0.03% NH_4Cl; 0.03% $MgSO_4$; 0.001% $FeCl_3$; 0.001% $CaCl_2$ and 0.1–0.3% of an organic carbon source. All the species studied by Doudoroff with the exception of $P.$ $phosphoreum$, emitted little or no light when grown on this medium.

In the present experiments, the salt water bacterium, $Achromobacter$ $fischeri$, was used exclusively and Doudoroff's medium was modified in the following manner. NaCl, 30 g.; $Na_2HPO_4 \cdot 12H_2O$, 4.5 g.; KH_2PO_4, 0.7 g.; NH_4NO_3, 1 g.; $MgSO_4$, 0.1 g.; glycerol, 10 ml.; trace elements, 0.05 ml.; L(+)-histidine, 10 mg.; DL-threonine, 10 mg.; and H_2O, 1 liter. The pH was adjusted to 7.3. A liter of the trace element solution contained the following: $CaCl_2$, 2.7 g.; $FeCl_3 \cdot 6H_2O$, 0.96 g.; $MnSO_4 \cdot 4H_2O$, 36 mg.; and $CuSO_4 \cdot 5H_2O$, 39 mg.

In this medium the bacteria grow and luminesce under certain conditions. Although histidine and threonine are not necessary for growth it was found that luminescence was much brighter and more consistent when the two amino acids were present. Subsequent studies have indicated that temperature is an important factor in growth and luminescence. At 23°C. $Achromobacter$ $fischeri$ grows and luminesces normally without histidine or threonine, whereas above 26°C. no growth occurs unless hydrolyzed casein is added. Unpublished observations of R. E. Anderson indicate that a mixture of L(+)-glutamic acid, DL-serine and DL-methionine can duplicate the effect of hydrolyzed casein at high temperature.

The complete medium was prepared as follows: basal medium, one liter; hydrolyzed casein, 200 mg.; vitamin mixture, 0.5 ml.; hydrolyzed yeast nucleic acid, 5 mg.; yeast

extract 0.5 g. and malt extract, 0.5 g. The vitamin mixture was the same as that previously described for *Neurospora* (10).

The mutants reported in the present study were obtained from suspensions of bacteria which had been irradiated with ultraviolet light from a Westinghouse "sterilamp," approximately 95% of the energy being emitted at a wavelength of 2537 Å. The bacteria were usually grown for 18 hours in the liquid basal medium plus 200 mg. of hydrolyzed casein per liter. Five to ten ml. of this suspension were then placed in a quartz flask at a distance of 24 inches below the mercury tube and rotated at a moderate speed during irradiation. After various time intervals samples were removed and the bacteria streaked on complete agar plates (23 g. nutrient agar to 1 liter of liquid complete). It was observed that the number of viable bacteria in such samples decreased rapidly subsequent to treatment with ultraviolet. For this reason the bacteria in a treated sample were plated immediately. The petri plates were incubated for 2 days at 23°C. At the end of this time well-isolated colonies were picked out and transfers made from each to a complete solid slant and a minimal liquid tube. The minimal cultures were incubated for 4 days at 23°C., after which time those which failed to grow were tested on minimal, minimal plus hydrolyzed casein, and minimal plus the vitamin mixture. In these tests 10 ml. of the minimal medium were used. The vitamin mixture in this case lacks vitamin K which was found to be inhibitory, even in very small concentration. In testing for vitamin and amino acid mutants a light suspension of the organism was made in 2 ml. of the minimal medium, and the test solutions were innoculated from this suspension by means of a platinum loop of approximately 0.003 ml. capacity. A similar procedure was used for testing individual amino acids and vitamins. All minimal tubes were observed in the dark to determine whether the capacity to luminesce had been lost.

For the quantitative study of growth and luminescence in the mutant forms, the organisms were grown in 125 ml. Erlenmeyer flasks. To these flasks 20 ml. of the basal medium and the desired concentration of the substance under investigation were added. After sterilization the flasks were inoculated from a light suspension using the platinum loop. The flasks were placed on a shaking machine (100 strokes/min. through a distance of 5 cm.) and incubated for 2–2.5 days at 23°C. The density of growth was measured on a Klett-Summerson photoelectric colorimeter and the light intensity by an electron multiplier tube (General Electric 931A). The wild type strain was always grown on the basal medium and was used as a basis of comparison.

RESULTS AND DISCUSSION

A detailed analysis of growth and luminescence has been made on only 5 of the various mutants obtained thus far with ultraviolet radiation. This group includes two mutants requiring arginine, one aspartic acid, one glutamic acid or proline, and one guanylic acid.

Fig. 1 shows the relationship between growth, luminescence, and arginine concentration for mutant 707. The results are expressed on a percentage basis considering the growth and luminescence of the wild type on the basal medium as 100. At low concentrations of arginine the

growth is essentially linear up to approximately 30% of the maximum density, then increases more slowly with increasing arginine concentrations. Light does not appear in a measurable amount until approximately 30% of the maximum density has been obtained. It would appear, therefore, that the block is concerned with both the luminescent and growth systems for it is seen from the graph that luminescence is not a necessary accompaniment of growth. Furthermore, the growth requirements are such that a concentration of arginine sufficient to give approximately 30% of the maximum growth must be available before the light system can function. This mutant appears to be an example
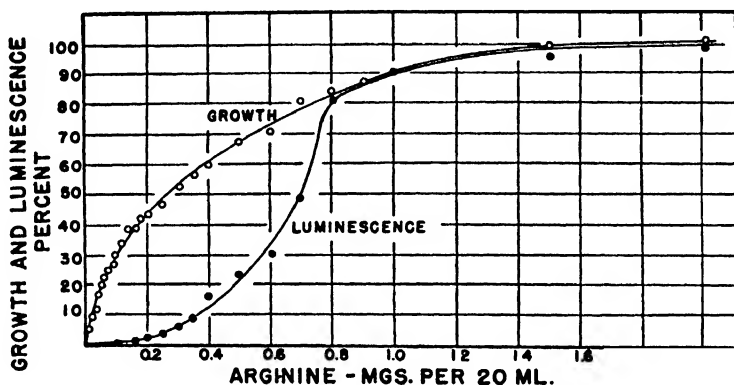
FIG. 1. The relationship between growth, luminescence and L(+)-arginine concentration for mutant 707. The results are expressed on a percentage basis considering the growth and luminescence of the wild type as 100. The growth period was 48 hours at 23°C.

of the type in which the light and growth systems compete for some common component. In this case arginine could be important as an amino acid in the synthesis of either the substrate, luciferin, or the enzyme, luciferase. Preliminary· tests indicate that yeast extract in concentrations insufficient to stimulate growth may be effective in removing the lag in the development of luminescence.

Over 20 amino acids including proline, hydroxyproline, L(+)-glutamic acid, DL-aspartic acid, DL-α-alanine, glycine, DL-tryptophan, L(−)-cystine, L(+)-cysteine, DL-serine, DL-lysine, L(−)-tyrosine, DL-methionine, DL-phenylalanine, L(−)-leucine, DL-isoleucine, L(+)-arginine and ornithine have been tested individually and in combination but, in addition to arginine, only ornithine supported growth.

Citrulline has not been tested. The growth and luminescence curve for ornithine is similar to that reported for arginine. The second arginine mutant appears to be identical with 707.

In Fig. 2 the relationship between growth, luminescence and concentration of DL-aspartic acid is shown for mutant 4639. The growth increases in a normal fashion with increasing concentration of aspartic acid and finally reaches the maximum density which is 10–15% higher than that of the wild type. Luminescence, on the other hand, is greatly
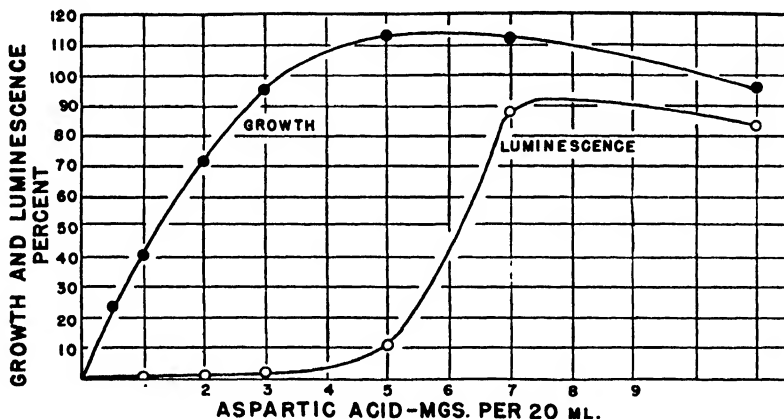
FIG. 2. The relationship between growth, luminescence and DL-aspartic acid concentration for mutant 4639. Density of growth and luminescence compared to wild type after 48 hours at 23°C.

delayed and, in fact, begins to appear in appreciable quantities only after maximum growth requirements have been satisfied. With increasing concentration of aspartic acid the luminescence increases rapidly to approximately 90% of the wild type.

In an attempt to increase luminescence at low concentrations of aspartic acid, we have tested various compounds of which only hydrolyzed casein appeared to increase luminescence. Upon testing all of the above mentioned amino acids individually, none were found which would increase the light intensity; however, several of the amino acids, in particular, L(−)-proline, L(+)-glutamic acid, and DL-valine, had a marked sparing action in relation to growth. From an investigation of various combinations of amino acids it was found that a mixture of DL-methionine, L(−)-leucine, DL-isoleucine and L(+)-

arginine would eliminate the lag in the development of luminescence. The relationship of luminescence to the concentration of this mixture is shown in Fig. 3. In this experiment 5 mg. of aspartic acid were added to the basal medium, a concentration which gives the maximum growth but very little luminescence. The mixture contained 1 mg. of
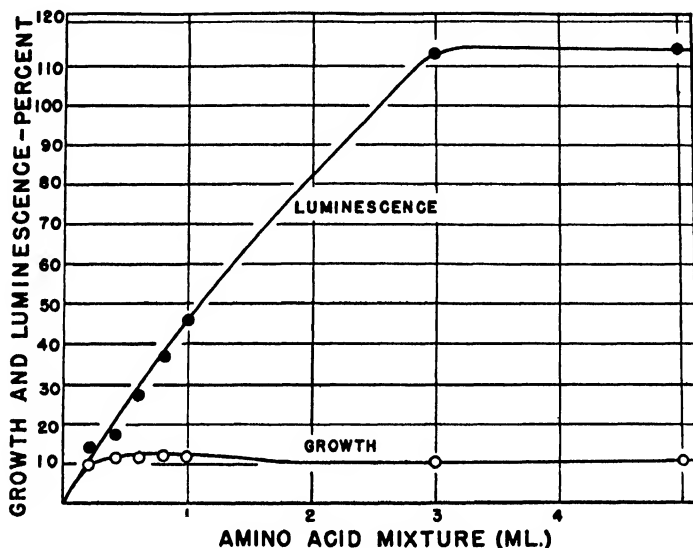
FIG. 3. The effect of an amino acid mixture containing 1 mg. each/ml. of DL-methionine, L(−)-leucine, DL-isoleucine and L(+)-arginine on growth and luminescence of mutant 4639. 5 mg. of DL-aspartic acid was added to 20 ml. of the basal medium to give maximum growth. The luminescence obtained upon the addition of the mixture was compared to the normal wild type intensity since no light was obtained with DL-aspartic acid. The growth density is compared to that obtained with DL-aspartic acid alone. See text for details.

each of the aforementioned amino acids/ml. The luminescence is compared to the normal wild type intensity and the growth compared to that obtained with 5 mg. of aspartic acid alone. At this concentration of aspartic acid the above mixture stimulates growth approximately 10%, whereas the luminescence surpasses the normal wild type intensity. If 5 ml. of the mixture are added to the basal medium and the aspartic acid concentration varied, there is very little lag in the development of the light intensity; the growth and luminescence curves now follow each other closely.

It is interesting that the elimination of any one of the 4 amino acids from the mixture prevents the production of any significant amount of light, irrespective of the concentrations of the remaining 3 amino acids. The combination of methionine, leucine and arginine gave the best results of the combination of three, but this was only 20% of the normal even with 5 mg. of aspartic acid in the minimal medium. Thus, there is no doubt that these 4 amino acids are necessary for normal development of light at concentrations of aspartic acid which just give the maximum growth. However, the 4 amino acids which stimulate the development of the luminescent system are not the amino acids which show a marked sparing action on the utilization of aspartic acid for growth. It is interesting, therefore, that concentrations of aspartic acid higher than that required for maximum growth also stimulate the development of luminescence. The results indicate that aspartic acid is only indirectly concerned in the light system.

When the aspartic acid mutant was originally tested for compounds which would support growth in the basal medium only aspartic acid was found to be active. However, subsequent tests revealed that glutamic acid in high concentration gives a small amount of growth, but, even with concentrations as high as 10 mg./20 ml. of basal, the maximum growth can not be obtained. With 5 mg. of glutamic acid only 10% of the normal density was obtained after 2 days of incubation. No growth could be obtained with glutamine. It has been reported by Stokes, Larsen and Gunness (13) that biotin is involved in the synthesis of aspartic acid in various strains of *Lactobacilli* and *Streptococci*. With our aspartic acid mutant, however, biotin was found to be completely inactive.

In contrast to the aspartic acid mutant, is strain 1473 which can use either proline or glutamic acid for growth but none of the other amino acids or α-ketoglutaric acid. The response to both glutamic acid and proline are very poor, however, giving at the optimum concentration of each only 30% of the normal. The nature of the growth response with glutamic acid is similar to that reported previously by other investigators for the utilization of glutamic acid by various strains of *Lactobacilli* (6, 8). In these organisms the sigmoidal nature of the growth curve could be completely eliminated by the addition of small amounts of glutamine to the assay medium. However, in the mutant 1473 addition of filter-sterilized glutamine (even in concentrations as high as 20 mg./20 ml. of basal medium) not only failed to remove the sigmoidal nature

of the growth curve but, by itself, gave only about 18% of the wild type growth. The sigmoidal nature of the growth curve is not eliminated with mixtures of glutamic acid and glutamine; however, with high concentrations of both, 70% of normal growth is obtained. These results suggest that, in this mutant, contrary to what occurs in *Lactobacillus*, glutamic acid and glutamine are not freely interconvertible. The combination of glutamic acid and proline is not much better than either alone. Other combinations are being studied.

Of particular interest, however, is the fact that luminescence fails to develop in this mutant, irrespective of the concentration of glutamic acid, proline or glutamine. In an attempt to induce luminescence in this strain, various components were tested, but so far no positive
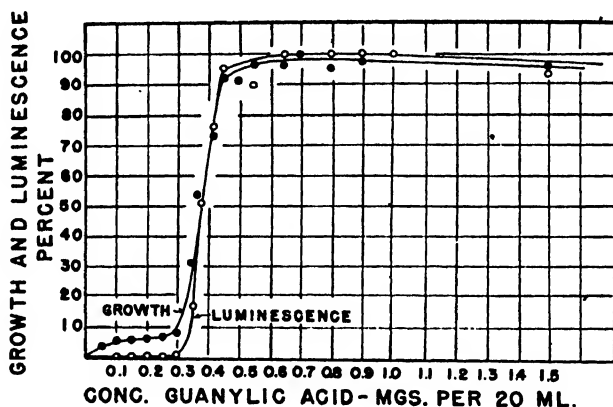
FIG. 4. The relationship between growth, luminescence and guanylic acid concentration for mutant 2633. Density of growth and luminescence compared to wild type after 48 hours at 23°C.

results have been obtained. It should be mentioned also that, even on complete agar slants, the luminescence is very much weaker than the wild type. It would appear that some complex component or mixture is essential for maximum growth and for the development of the luminescent system. This point is being investigated.

Mutant strain number 2633 requires guanylic acid for growth and differs from the previous mutants in that the luminescence system shows no appreciable lag in its development with respect to growth. However, there is a considerable delay in growth response to increasing concentrations of guanylic acid. The results are summarized in Fig. 4.

Approximately 5% of the normal growth is obtained with 0.3 mg. guanylic acid/20 ml. of basal medium. With increasing concentrations of substrate above this level the growth develops rapidly and reaches its maximum at approximately 0.5 mg. Except at very low concentrations of guanylic acid, the luminescence parallels the growth curve. These results suggest that guanylic acid itself is not the component primarily concerned in the growth-limiting process but must first be converted into some functional cellular component. This interpretation is similar to that offered for the utilization of glutamic acid by *Lactobacilli* (6, 8). Furthermore, these results seem to indicate that guanylic acid itself is not primarily concerned with the luminescent system because, with the initiation of growth, the functioning of the systems concerned with light production are restored and the luminescence parallels the growth curve.

In addition to the usual vitamins and amino acids the above mutant has been tested on thymine, cytosine, xanthine, uracil, adenine, adenosine, adenosine 3- and 5-phosphoric acid, hypoxanthine, guanine, and guanosine. Of this group only guanosine initiates growth. The growth on this substance, however, is very poor, giving, at the optimum concentration of 0.5 mg./20 ml. of basal medium, only 20% of the normal. Higher concentrations are quite inhibitory to both growth and luminescence.

DISCUSSION

The results of the present study concerning the relationship of light production and growth in mutant strains of luminous bacteria emphasize the fact that other physiological functions may not necessarily be fully restored with concentrations of growth factors sufficient to give maximum growth. However, it would appear that in some respects the growth and luminescent characteristics of the mutants are merely an accentuation of those which normally occur in the wild type strain during the early phases of rapid growth. During this stage in the rapid growth of the wild type there is an appreciable lag in the development of the luminescence. Subsequent to this lag, however, the luminescence develops rapidly and thereafter keeps pace with the growth. The reason for the discrepancy between growth and luminescence in the wild type during the first few hours is at present not clear. It would appear possible, however, that, with the initiation of rapid growth, there is either a lag in the synthesis of the components essential for

maximum luminescence or an excessive demand upon components common to the two systems.

In certain biochemical mutants it has actually been possible to demonstrate an apparent competition of two systems for the added growth substances. This is true for the aspartic acid mutant which produces very little light even at concentrations sufficient to give the maximum growth response, regardless of whether the comparison to the wild type is made at 24, 48, or 72 hours of incubation. Furthermore, in some mutants it would appear that, at concentrations of growth substances inadequate to support normal growth, the light intensity never attains its maximal value, irrespective of the time of incubation. This point is made clear by reference to Fig. 2. It is apparent that, at concentrations just below that for optimal growth, the light intensity is less than 5% of the maximum. In the case of the aspartic acid mutant the lag in luminescence can be eliminated by the simultaneous addition of methionine, leucine, isoleucine, and arginine. All 4 amino acids are apparently necessary, since any combination of 3 fails to eliminate the lag in luminescence. Thus, it would appear that there are two possible ways by which the development of the light system may be controlled. These two possibilities are seen in the mutant wherein aspartic acid can be made limiting for growth. Under these conditions it becomes evident that other factors are required for luminescence. Since normal luminescence develops at aspartic acid concentrations greater than those necessary to satisfy the maximal growth, and since none of the 4 amino acids replaces the aspartic acid requirement of this mutant nor manifests a great sparing action (either separately or in combination), it would seem clear that the elimination of the lag in luminescence by the addition of these amino acids cannot be explained on the basis of a decrease in their synthesis by the mutant. An indirect effect of these amino acids on luminescence seems to be most likely. Perhaps under exacting growth conditions, i.e., aspartic acid deficiency, supplements of these amino acids restore or stabilize in some fashion the normal functioning of a system, i.e., luminescence, in which they are not directly involved. How this may occur is not clear, but the effect does have certain features common with those observed in studies of growth antagonists and their reversal.

An extensive analysis at various time intervals of development has not been made, but it might be expected that such a study would reveal even greater differences between the mutants and wild type

than reported above. In some cases the mutants have been incubated for longer periods of time (5–6 days) and the maximum growth and luminescence of the mutant considered as 100%. The results in all cases were, however, essentially the same as those discussed previously where the growth was compared to the wild type.

It is possible that, with further isolations of mutants in luminous bacteria, we can obtain a strain which grows normally on the minimal medium but luminesces only on the complete, *i.e.*, a specific block concerned only with light production. Thus far such a mutant has not been obtained. Although the number of our isolations is too few to justify a definite conclusion concerning the feasibility of obtaining such a mutant, it might be expected from other considerations that the normal substrate of the light system, luciferin, is also concerned with the growth of the organism. If this is true, one would not expect to obtain a block limited to the light system, at least by disrupting the synthesis of the substrate.

Summary

1. Biochemical mutations which affect both growth and luminescence have been obtained in the luminous bacterium, *Achromobacter fischeri*, by treatment with ultraviolet light.

2. In a strain which requires arginine for growth, the luminescence fails to develop in growing cultures until the concentration of the arginine is high enough to give 30% of the normal growth. With higher concentrations of arginine, the luminescent system develops rapidly and finally reaches the wild type level of intensity at a concentration which just gives the maximum growth.

3. In an aspartic acid mutant luminescence develops only after the maximum growth has been attained. After the growth requirements have been satisfied, the luminescent system develops rapidly with increasing concentrations of DL-aspartic acid, finally reaching 90% of the wild type level of intensity. Supplements of a mixture of methionine, leucine, isoleucine, and arginine will prevent this lag in the development of the luminescent system.

4. In a mutant requiring guanylic acid there is a lag in both growth and luminescence until a critical concentration is reached, beyond which point both growth and luminescence increase rapidly and attain their maximum value within a narrow concentration range. Guanine fails to support growth and guanosine gives, at the optimum concentration, only 10% of the normal growth and luminescence.

5. In one mutant either glutamic acid, glutamine or proline could support growth but only 20–30% of the normal density could be obtained with these compounds. A mixture of glutamine and glutamic acid increased growth to 70% of the normal. Luminescence fails to develop, irrespective of the concentrations of glutamic acid, glutamine or proline. Glutamine fails to remove the sigmoidal nature of the growth curve.

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On the Mechanism of Histamine Formation

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INTRODUCTION

It was shown in earlier experiments (1, 2) that histidine derivatives, acylated in the amino group (*e.g.*, acetyl- and benzoylhistidine) are not attacked by bacterial decarboxylases. Enzymatic decarboxylation was, therefore, regarded as a highly specific reaction, since it may be blocked by substitution in the amino group.

The question of whether or not bacteria can convert peptides into histamine-containing "peptamines" by decarboxylation, as suggested by Guggenheim (3), was, however, even by these experiments, undecided. There remained the possibility that the requisite free α-amino groups could also be supplied by peptides containing histidine units with free carboxyl groups, as well as by histidine *per se*. The negative results obtained in studies on the decarboxylation of carnosine (4) are not quite pertinent to the present question, since this naturally occurring peptide contains the free amino group of β-alanine, instead of the essential α-amino group.

We, therefore, decided to investigate enzymatic decarboxylation of 2 histidine-containing peptides: aspartyl-histidine, containing histidine with a free carboxyl group; and histidyl-histidine, with both a free carboxyl and free amino group on the histidine residues.

METHODS

The β-L($-$)-aspartyl-L($-$)-histidine was obtained through the courtesy of Dr. V. du Vigneaud (5); the L($-$)-histidyl-L($-$)-histidine was prepared in our laboratory according to the methods described by Pauly (6) and Fischer-Suzuki (7), with methylhistidine and histidine anhydride as intermediate stages.

Three methods for decarboxylation were attempted: First, with an enzyme preparation derived from *Cl. welchii* and generously supplied by Dr. E. Gale of Cambridge University; second, with a strain of *E. coli* isolated from human feces; and third, with marine bacteria isolated from ocean-fresh Pacific pilchards. Control experiments have shown that both the marine bacteria and the *coli* are very effective in histamine formation from histidine.

Both strains were grown in 500 ml. of tryptose-broth, pH 5.8, at 30°C. for 16 hours. The media for the marine bacteria were prepared with aged sea-water. The bacteria were then centrifuged, washed twice, and finally suspended in 10 ml. of sterile saline. To this supension were added 50 mg. of the substrate in 10 ml. H_2O and 10 ml. of $M/10$ phthalate buffer (pH 5.0). These samples, and blanks containing no bacteria, and others containing no substrate, were incubated at 37°C. for 10 hours. The enzyme preparation from *Cl. welchii* was used in accordance with the instructions of Epps (8).

Histamine formation was assayed by both response of the isolated guinea pig-ileum and blood pressure change after i.v. injection into nembutalized cats.

RESULTS

I

No free histamine was formed in any of the experiments where the peptides were incubated with bacteria or with the enzyme preparation, whereas parallel control samples using L-(−)-histidine as a substrate showed considerable histamine formation.

Since it is known that amine-substituted histamine compounds are biologically inactive (2 and 9), there was a possibility that the peptides had been decarboxylated, but that the activity of the histamine unit formed was masked by peptide linkages involving the amino groups. This question was investigated by hydrolyzing the incubated samples with 10% HCl for 6 hours on a boiling water bath, followed by evaporation of the HCl *in vacuo*, neutralization, and redetermination of the biological activity. *None of the hydrolyzed samples showed any histamine action, so that the possibility of formation of histamine in peptide linkage could be discarded.*

In the light of a recent claim (10) that racemization of histidine occurs during the preparation of histidyl-histidine, we investigated the extent to which the histidine unit in the peptides was susceptible to enzymatic decarboxylation. Samples of histidyl- and aspartyl-histidine were hydrolyzed with N H_2SO_4 for 4 hours in a boiling water bath. The acid was then removed with $Ba(OH)_2$ and finally concentrated *in vacuo* to 10 ml. These hydrolyzates were then incubated with the bacteria and enzyme preparations following the procedures given above. In all cases *considerable quantities of histamine were produced*

TABLE I

Histamine Production from Acid-Hydrolyzed Peptides

Substrate	Incubated with	Mg. histamine produced
50 mg. Acid-hydrolyzed histidyl-histidine	*E. coli*	22.4
	Marine bacteria	21.6
	Enzyme preparation[a]	18.5
50 mg. Acid-hydrolyzed aspartyl-histidine	*E. coli*	11.5
	Marine bacteria	14.6
	Enzyme preparation[a]	9.4

[a] The amount of histamine produced by the enzyme preparation is relatively low, probably because the preparation lost activity during transportation and storing in our laboratory.

(*cf.* Table I), indicating that significant quantities of L-(−)-histidine groups were present in the original peptides, and that their decarboxylation had been prevented by the peptide linkages. This conclusion is in agreement with the statements of Werle (11), Gale (12), and Epps (8), who consider an unsubstituted amino group in the substrate as essential for enzymatic decarboxylation.

To establish conditions closer to those under which bacteria normally attack proteins, we repeated the experiments, incubating the peptides with the bacteria at the slightly alkaline pH of 7.2. No histamine was produced, indicating that the histidine in peptide linkage withstands enzymatic decarboxylation in alkaline as well as acid media.

II

The preceding experiments show that peptide linkage to either the —COOH or —NH₂ group of histidine inhibits bacterial decarboxylation. The possibility of decarboxylation of histidine in peptides with amino acids other than alanine, aspartic acid, or another histidine, was not, however, eliminated. We therefore investigated whether some of the histamine produced by bacterial action on protein is present in the form of peptides. Since histamine substituted in the amino group shows no biological activity, but can be restored to an active form by acid hydrolysis, we assumed that, if some significant part of the histamine formed were present in peptide linkage, a difference in the assay should be apparent between samples taken before and after acid hydrolysis.

Proteins for these experiments were prepared from fish and rabbit muscle, rabbit liver, and from milk by fairly aseptic techniques, using isoelectric precipitation and solvent extraction. Free histamine possibly present in the protein preparations was

removed by the extraction method described by McIntire *et al.* (13). Five g. of very finely ground protein were homogenized with 50 ml. of water. After addition of 3 g. of the alkaline buffer salt mixture, the suspension was extracted 6 times with 25 ml. aliquots of normal butanol. The filtered protein was then thoroughly washed with distilled water and dried at low temperature *in vacuo*. One gram of the extracted, finely powdered protein was suspended in 7 ml. saline, and the pH of the suspension brought to pH 6.7 with saturated Na_2HPO_4, and the volume then brought to 10 ml. with saline. To this suspension were added 10 ml. of 5% glucose solution and 10 ml. of the bacterial suspension. After 24 hours incubation at 30°C., with repeated shakings, the samples were divided into 2 parts. The histamine content of the first portion was measured directly, after dilution with saline; control experiments had shown that the presence of some suspended proteins in the dilution did not interfere with the quantitative determinations of histamine. The second portion was hydrolyzed with 10% HCl for 8 hours, with refluxing. After removal of the HCl *in vacuo*, the sample was neutralized, brought to the original volume with distilled water, and the histamine content determined.

Table II presents the data obtained and indicates that *acid hydrolysis did not increase the histamine content of the incubated samples*. The biologically-determined histamine concentrations are the same within

TABLE II

Histamine Production from Protein
(Mg. histamine/100 g. protein)

Protein	Before incuba-tion after acid hydrolysis	After incubation		Incubated with
		Determined directly	After acid hydrolysis	
Casein	>0.1	45	Same	*E. coli*
Fish protein	>0.02	76 120	Same Same	*E. coli* Marine bacteria
Rabbit muscle	>0.01	53	Same	*E. coli*
Rabbit liver	>0.07	45	Same	*E. coli*

the limits of experimental error. These results indicate that none of the histamine produced by bacterial action on protein was present in the peptide form.[1]

[1] Our earlier observations and our assumptions concerning the existence of such bound histamine (1) must be reconsidered, since, by use of McIntire's method, it is possible to remove practically all of the histamine from the protein samples studied.

In view of these experiments, possible differences between the decarboxylases of animal tissues (11, 14) and those derived from bacteria should be investigated. Until such a mechanism is demonstrated experimentally there seems to be no direct evidence for the assumption that histamine may occur in the blood or tissues in the form of inactive peptides (9).

SUMMARY

1. It has been shown that peptides containing histidine units with free carboxyl groups are not decarboxylated by bacteria.

2. The histamine activity of protein suspensions and casein solutions incubated with bacteria is not increased by acid hydrolysis.

It is concluded that histamine produced by bacterial decarboxylation is not present in the form of inactive "peptamine."

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Protein Intake and Vitamin B₆ Deficiency in the Rat. II. The Effect of Supplementing a Low-Protein, Pyridoxine-Deficient Diet with Cystine or with Methionine [1]

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INTRODUCTION

In a previous paper (3), we reported that the protein level in the diet has a pronounced effect on the development of the symptoms of vitamin B₆ deficiency in the rat. In rats receiving diets high in casein, the acrodynia appeared at an earlier period, and was more severe than in those fed diets low in casein. The aggravating effect of high-protein diets on the development of the vitamin B₆ deficiency syndrome has also been observed by Miller and Baumann in the mouse (4). In contrast to our findings, these workers reported that a high level of casein in the diet was less harmful in the rat than in the mouse. It is to be noted, however, that in their experiments Sprague-Dawley rats were used (see below). The detrimental effect of a high-protein diet in the vitamin B₆-deficient dog has been observed by Morgan *et al.* (5).

The experiments to be discussed below were begun with the idea in mind that some amino acid (or acids) might be responsible for the harmful effect caused by the higher level of protein. Since casein is low in the sulfur amino acids, it was decided to investigate first the effect of a low-protein diet supplemented with cystine. The results obtained with this amino acid led us to study the effect of methionine. Lastly, since methionine sulfoxide is considered to be an intermediate of methionine metabolism (6), a diet containing this compound was also

fed to the rats. While preparing the manuscript of this paper, we learned of the work of Sakurai and Aoyagi (7), who also observed the harmful effect of high-protein diets in vitamin B_6 deficiency in the rat. Of greater interest, in connection with the present work, was their finding that cystine, when added to a low-protein diet, had a detrimental effect.[5]

EXPERIMENTAL

In studies on vitamin B_6 deficiency in the rat, certain precautions must be observed. The first is that there are strain differences in the resistance to the deficiency (2). In our experience, Wistar rats give the best results. Secondly, it is essential to use animals weighing not more than 35 g. when placed on the experimental diets. If heavier animals are used, the storage factor comes into play (9).

In this study, 7 experimental diets have been used. The composition of 4 of the diets is given in Table I. The other 3 diets differed from these only in the content of

TABLE I

Composition of the Diets

Constituents	Diet			
	P-3(a)	P-6(a)	P-7(a)	P-7(a)SO
Purified casein[a]	15	15	15	15
Sucrose	70	69.5	69.69	69.66
Hydrogenated vegetable fat[b]	5	5	5	5
Salts[c]	7	7	7	7
Cod liver oil	3	3	3	3
L-Cystine	—	0.5	—	—
DL-Methionine	—	—	0.31	—
Methionine sulfoxide[d]	—	—	—	0.34
Vitamin supplements added/kg. of diet:				
Thiamine (mg.)	4	4	4	4
Riboflavin (mg.)	4	4	4	4
Calcium pantothenate (mg.)	20	20	20	20
Choline chloride (g.)	1.0	1.0	1.0	1.0

[a] Labco or Smaco.

[b] Crisco or Primex.

[c] Osborne and Mendel (16) or Hawk and Oser (17). The amount of $MnSO_4$ was doubled.

[d] Prepared according to the method of Toennies and Kolb (18).

[5] Sarma *et al.* [*J. Nutrition* **33**, 121 (1947)] have recently shown that DL-methionine has a growth-retarding effect when added to a diet deficient in pyridoxine. Contrary to our observations, L-cystine was found to have no appreciable effect.

3 of the B vitamins. They contained/kg.: Thiamine, 10 mg.; riboflavin, 10 mg.; and Ca pantothenate, 40 mg. Similar results were obtained on either level of these factors. The majority of the rats used in our experiments were males. They came, with few exceptions, from mothers that were on Purina Dog Chow or Rockland diets.

The results of our experiments are summarized in Table II. The animals that received the unsupplemented low-protein diet were quite resistant to the deficiency. Five of the rats in this group had been on the experimental ration 154, 158, 183, 193,

TABLE II

Data Showing the Deleterious Effects of Cystine,
Methionine, and Methionine Sulfoxide

Diet	No. of rats	Average initial weight	Time of appearance of first symptoms of the acrodynia	Survival time
		g.	*Av. in days*	*Av. in days*
P-3(a)	12	34	57	*a*
P-6(a)	13	32	18	56
P-7(a)	14	30	17	41
P-7(a)SO	9	30	25	44

a With the exception of 3, all animals in this group lived more than 100 days.

and 193 days, respectively, when they were taken off the diet. Thus, these results confirm our previous findings. Addition of cystine or methionine to the low-protein diet had an aggravating effect on the development of the deficiency. Of the two amino acids, methionine seems to have a more pronounced effect. This is also borne out by the results obtained with methionine sulfoxide. Occasionally, a rat will show itself to be refractory to the deficiency. Data on such animals have not been included in the table.

The possibility that the lack of biotin or of pteroylglutamic acid in the diets might have influenced the results has been ruled out by supplementing our diet P-6 (a) with 10 mg. of pteroylglutamic acid and 100 γ of biotin/kg. of diet. No difference in the resistance of the animals given this diet as compared with those receiving the unsupplemented diet was observed.

On the basis of these findings the conclusion seems to be justified that in vitamin B₆ deficiency there is a disturbance of the metabolism of the thio amino acids.

DISCUSSION

That vitamin B₆ may play a role in the metabolism of other amino acids is indicated by observations reported from several laboratories. Thus, Fishman and Artom (10) found that, among the B vitamins,

pyridoxine was the most effective in protecting rats against the toxic effects of DL-serine. A harmful effect of glycine in vitamin B_6-deficient rats was reported by Pagé and Gingras (11). Schlenk and Snell (12) observed that tissues from such animals exhibited a marked drop in their ability to catalyze a transamination reaction, and that pyridoxal plus adenosine triphosphate produced a maximum restoration of this activity.

Observations on bacteria and higher plants also point to a function of vitamin B_6 in amino acid metabolism. Umbreit *et al.* (13) found that "pyridoxal phosphate" acts as a coenzyme in the synthesis of tryptophan from indole and serine by *Neurospora sitophila*. Previous investigations in the same laboratory (14–19) had shown pyridoxal and "pyridoxal phosphate" to act as coenzymes for several bacterial apodecarboxylases. This work has been confirmed by Baddiley and Gale (20). A coenzyme effect of "pyridoxal phosphate" in the decarboxylation of glutamic acid in higher plants was observed by Schales *et al.* (21).

Snell and Guirard (22) found that large amounts of alanine replaced pyridoxine as a growth factor for *Streptococcus lactis* R. Using cell-free preparations of this organism, Lichtstein *et al.* (22), showed that "pyridoxal phosphate" acted as a coenzyme in transamination reactions.

The data presented in this paper point to another function of vitamin B_6 in the mammalian organism, *viz.*, as a factor governing the normal metabolism of the thio amino acids. That this vitamin may exert a similar function in bacteria is indicated by the work of Speck and Pitt (24).

In trying to explain the deleterious effect of the sulfur amino acids on the development of the vitamin B_6 deficiency syndrome in the rat, the idea suggests itself that there may be a connection between the integrity of the epidermis and the normal metabolism of cystine and methionine. Such an interrelation might be expected if we are dealing with a tissue, the essential component of which contains a rather high cystine content. In this connection, it may be significant that, in contrast to the rat, the mouse does not develop the typical acrodynia when given a vitamin B_6-free diet (25, 26). The lesions develop in the mouse, however, when such diets contain desoxypyridoxine (27). Here also a high protein level has a detrimental effect.

SUMMARY

Rats fed a low-protein, vitamin B₆-deficient diet were found to be quite resistant to the deficiency.

Addition of cystine or methionine to such a diet decreases the resistance. A similar effect was observed when methionine sulfoxide was added.

These findings justify the conclusion that in vitamin B₆ deficiency there is an abnormal metabolism of cystine and methionine.

A possible connection between the integrity of the epidermis and the metabolism of the thio amino acids is pointed out.

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The Effect of BaCl₂, MgCl₂ and Fluoroacetate on the Formation and Utilization of Citrate

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INTRODUCTION

Various tissues can synthesize citrate from oxaloacetate, or from oxaloacetate plus a number of other compounds such as acetate, pyruvate, acetoacetate, and other β-keto acids (see reviews by Krebs (1) and Wood (2)). Citrate formation is influenced by various metallic ions. Barium acetate was found to be more effective than the other metallic acetates for the formation of citrate by brewers' yeast (3, 4), whereas magnesium acetate was slightly superior to barium acetate (5) in this respect. BaCl₂ was as effective as Ba acetate in the formation of citrate from oxaloacetate and acetoacetate by kidney (6).

The present communication describes the effects of BaCl₂ and MgCl₂ in augmenting citrate formation from oxaloacetate by a rabbit kidney cortex homogenate. BaCl₂ is not as effective as MgCl₂, and is inhibitory at certain concentrations. The increased citrate formation in the presence of these metallic ions can be accounted for by inhibition of citrate utilization. Sodium fluoroacetate inhibits acetate utilization by various tissues (7, 8) and inhibits citrate formation from acetate by yeast (8). This report demonstrates that sodium fluoroacetate increases the yield of citrate from oxaloacetate, whereas the other halogen acetates are inactive. Citrate utilization is somewhat inhibited at higher concentrations of fluoroacetate, but, unlike the situation with the metallic ions, this cannot entirely account for the effect of fluoroacetate in increasing the yield of citrate from oxaloacetate.

METHODS

Rabbits were stunned by a blow on the head, bled, and the kidneys removed immediately. The kidney cortex, plus Krebs-Ringer bicarbonate solution (9), was ground in a Potter-Elvehjem glass homogenizer (10), to give a final concentration of

175 mgs. wet weight tissue/ml. The ground tissue (generally 350 mgs. wet weight tissue per vessel) was incubated for 60 minutes in a 50 ml. Erlenmeyer flask, containing 5 ml. total volume. The flasks were shaken at 100 or 120 three and one-half centimeter strokes/min. at room temperature, generally 28–30°C. The tissue was incubated for 10 minutes with Ba^{++}, Mg^{++}, or fluoroacetate before the addition of the substrate.

Citrate was determined according to the method of Pucher *et al.* (11) with modifications described by Speck, Moulder and Evans (12). The preliminary treatment with bromine was eliminated after many experiments showed that this simplification was permissible. The extractions were carried out in 15×150 mm. pyrex test tubes, with rubber stoppers. A sodium sulfide-glycerol reagent was used to stabilize the color (13). The color was determined in the Coleman Universal Spectrophotometer, at 440 mμ. At least 2 standard citrate samples were run simultaneously with each group of determinations.

Oxaloacetic acid was prepared according to Krampitz and Werkman (14) and was dissolved and neutralized immediately before use.

Sodium monofluoroacetate was obtained through the courtesy of Dr. J. O. Hutchens, Director, the University of Chicago Toxicity Laboratory.

Results

Effect of $BaCl_2$ and $MgCl_2$ on Citrate Formation

Ground rabbit kidney cortex, in the presence of oxaloacetate, produced small amounts of citrate. The yield of citrate was increased on

TABLE I

Effect of $BaCl_2$ and Fluoroacetate on Citrate Formation From Oxaloacetate

Conditions	Fluoroacetate	$BaCl_2$ conc.	Citrate formed
		M	μM
Blank	—	—	0.2
Oxaloacetate	—	—	2.7
Oxaloacetate + $BaCl_2$	—	0.008	5.6
Oxaloacetate + $BaCl_2$	+	0.008	8.5
Oxaloacetate + $BaCl_2$	—	0.016	5.4
Oxaloacetate + $BaCl_2$	—	0.024	4.1
Oxaloacetate + $BaCl_2$	+	0.024	4.9
$BaCl_2$	±	0.024	0.1
Oxaloacetate	+	—	12.3
—	+	—	2.1

Each vessel contained tissue homogenate, 350 mgs. wet weight; oxaloacetate, 120 μM (0.024 M); $BaCl_2$ in indicated concentrations; fluoroacetate, 0.01 M; total volume, 5.0 ml.; air; 60 mins.

the addition of 0.008 M BaCl$_2$, and still further increased in the presence of both BaCl$_2$ and fluoroacetate (Table I). Larger concentrations of BaCl$_2$ had an inhibitory effect. The largest yields of citrate were obtained in the presence of oxaloacetate plus fluoroacetate, with no BaCl$_2$ present (Table I). Fluoroacetate alone, in the absence of tissue, gave no test for citrate. However, fluoroacetate and tissue resulted in a small blank citrate formation.

BaCl$_2$ and MgCl$_2$ were compared as to their effects on citrate formation from oxaloacetate by this kidney homogenate (Table II). At

TABLE II

Effect of Tissue Concentration, BaCl$_2$, and MgCl$_2$ on Citrate Formation from Oxaloacetate

Tissue	μM citrate formed			
	BaCl$_2$	BaCl$_2$ + oxaloacetate	MgCl$_2$	MgCl$_2$ + oxaloacetate
mgs. wet weight				
150	0.0	1.5	0.0	3.2
200		2.2		5.4
250	0.0	3.2	0.6	7.6
300		3.7		8.5
350	0.0	4.4	0.7	10.0
400	0.0	5.7	0.8	11.5

Tissue, in indicated amounts; oxaloacetate, BaCl$_2$, MgCl$_2$, 100 μM (0.02 M); total volume, 5.0 ml.; air; 30 mins.

various tissue concentrations, MgCl$_2$ was at least twice as effective as BaCl$_2$, and about as effective as magnesium acetate.

Effects of Halogen Acetates on Citrate Formation

Whereas fluoroacetate resulted in a pronounced increase in citrate formation, chloroacetate did not have this effect (Table III). On the contrary, there was a 40% inhibition of citrate formation, in the presence of 0.005 and 0.01 M chloroacetate. Approximately the same held true for iodoacetate and bromoacetate, the inhibitions obtained being 64–68%. Sodium fluoride had little or no effect, as has been reported by previous investigators (15, 16). This further demonstrates the relative nonreactivity of fluoroacetate as compared to the inhibitory

TABLE III

Effect of Halogen Acetates and Sodium Fluoride on Citrate Formation from Oxaloacetate

Halogen acetate	Conc.	μM citrate formed	
		Blank	Oxaloacetate
	M		
—	—	0.2	2.7
Fluoroacetate	0.01	2.1	12.8
Chloroacetate	0.01	0.0	1.5
Chloroacetate	0.005		1.5
Iodoacetate	0.01	0.2	1.0
Iodoacetate	0.005	0.1	1.0
Bromoacetate	0.01	0.0 ·	0.9
Sodium Fluoride	0.01	0.2	2.5

Tissue, 350 mg. wet weight per vessel; oxaloacetate, 100 μM (0.02 *M*); halogen acetates in indicated concentrations; 5.0 ml.; air; 60 mins.

effects obtained with the other halogen acetates, and the apparent specificity of fluoroacetate in this reaction.

Comparative Effects of MgCl₂, BaCl₂ and Fluoroacetate at Various Concentrations

The effects of Mg^{++} and fluoroacetate in increasing citrate formation were strikingly similar, and somewhat different from the effects produced by Ba^{++}. This seemed to hold over a wide range of concentrations (Fig. 1). The effect with Mg^{++} is easily discernible at a concentration of 6–8×10^{-4} *M* and seems to level off at a concentration of 2×10^{-2} *M*. The effect with fluoroacetate seems somewhat greater. However, if the blank values are subtracted for citrate formation in the presence of $MgCl_2$ or fluoroacetate, the curves obtained are practically identical.

The results obtained with Ba^{++} were quite different. Ba^{++} was generally not as effective as Mg^{++} or fluoroacetate, and had alternate stimulating and inhibiting effects at increasing concentrations, with an optimum stimulation at 7–8×10^{-3} *M*. These results are difficult to interpret. However, somewhat similar effects are known. Mn^{++}, Mg^{++} (17), and Fe^{++} are stimulants for the carboxylase system of

yeast, whereas Zn^{++}, Ca^{++}, Ni^{++}, and Co^{++} retard in small concentrations and stimulate in higher concentrations (18), but at still higher concentrations, stop the reaction entirely and irreversibly.
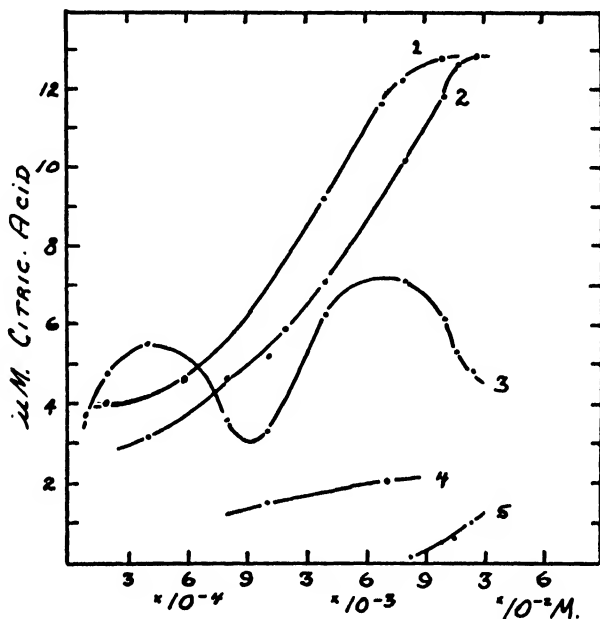
FIG. 1. The effect of varying concentrations of $BaCl_2$, $MgCl_2$ and fluoroacetate, on citric acid formation. Curve 1, oxaloacetate plus fluoroacetate; Curve 2, oxaloacetate plus $MgCl_2$; Curve 3, oxaloacetate plus $BaCl_2$; Curve 4, fluoroacetate; Curve 5, $MgCl_2$; $BaCl_2$. Tissue, 350 mg. wet weight per vessel; oxaloacetate, 100 μM (0.02 M); $BaCl_2$, $MgCl_2$, fluoroacetate, in indicated concentrations; total volume, 5.0 ml.; air; 60 mins.

Effect of $BaCl_2$ and $MgCl_2$ on Citrate Utilization

The addition of either $BaCl_2$ or $MgCl_2$ resulted in an inhibition of citrate utilization, Mg^{++} being more effective than Ba^{++} in this respect (Table IV). The same effects were obtained with lower concentrations of citrate (20 μM).

It appears that the inhibition of citrate utilization in the presence of these ions can adequately account for the increased citrate formation from oxaloacetate (compare Fig. 1 and Table IV). For example, the increased citrate formation in the presence of 0.01 M Mg^{++} is 8.8 μM (Fig. 1); in the presence of the same concentration of Mg^{++}, 8.6 μM

TABLE IV

Effect of MgCl₂ and BaCl₂ on Citrate Utilization

Conditions	μM Citrate		Per cent inhibition
	Remaining	Utilized	
Control	30.1	—	—
Citrate	14.5	15.6	—
Citrate + MgCl₂	23.1	7.0	55.1
Citrate + BaCl₂	21.1	9.0	42.3

Tissue, 350 mg. wet weight per vessel; citrate, 30 μM; MgCl₂, BaCl₂, 0.011 M; air; 60 mins.

less citrate are utilized (Table IV). With Ba^{++}, approximately the same picture is obtained.

Effect of Fluoroacetate on Formation and Utilization of Citrate

At concentrations of fluoroacetate (0.001 M) which completely inhibited acetate oxidation by this preparation, citrate utilization was only slightly inhibited (11.9%). Higher concentrations of fluoroacetate inhibited citrate utilization to a somewhat greater extent, and also resulted in an increased yield of citrate from oxaloacetate. However, the inhibition of citrate utilization (Table V, Col. 5) only accounted

TABLE V

The Effect of Varying Concentrations of Sodium Fluoroacetate on Formation and Utilization of Citrate

Conditions	Fluoro-acetate conc.	Citrate formed from oxaloacetate	Increased citrate due to fluoroacetate	Inhibition of citrate utilization	
	M	μM	μM	μM	*Per cent*
Aerobic	—	2.2	—	—	—
	0.001	5.5	3.7	1.7	11.9
	0.005	7.8	5.6	3.5	24.5
	0.01	10.0	7.8	4.6	31.7
Anaerobic	—	3.2	—	—	—
	0.02	6.2	3.0	0.0	0.0

Tissue, 350 mg. wet weight per vessel; citrate, 20 μM; oxaloacetate, 100 μM (0.02 M); fluoroacetate, in indicated concentrations; phosphate buffer, pH 7.4, 0.02 M; total volume, 5.0 ml.; 60 mins.; atmosphere, aerobic experiments, air; anaerobic experiments, N₂.

for 46–63% of the increased citrate formed from oxaloacetate (Table V, Col. 4). Anaerobically, citrate utilization was markedly decreased (3–5 μM). The addition of fluoroacetate did not inhibit citrate utilization under these conditions, while it did slightly increase citrate formation from oxaloacetate. These results indicate that, unlike Mg^{++} or Ba^{++}, the effect of fluoroacetate in augmenting citrate formation is not entirely due to inhibition of citrate removal.

DISCUSSION

It is probable that citrate formation from oxaloacetate is a side reaction, arising from cis-aconitate (see review by Wood (2)). In the equilibrium, citrate $\rightleftarrows$ cis-aconitate $\rightleftarrows$ isocitrate, approximately 89% of these compounds exist in the form of citrate (19, 20). High concentrations of $MgCl_2$ (0.12 M) further shift the equilibrium to approximately 96% citrate (20, 21). However, this probably does not account for the effects obtained with Mg^{++} in this report, since concentrations of 0.01 M $MgCl_2$ have no appreciable effect on aconitase (22). Since Mg citrate is more soluble than Ba citrate, solubility cannot account for the superior effect of Mg^{++} over Ba^{++} in citrate formation (5). The formation of soluble Mg citrate complexes which cannot penetrate the cell membrane, has been postulated (23). The toxicity of these cations or the lack of availability to the activating system may be a factor. Mg^{++} may also be a part of the citrogenase enzyme system.

The results obtained with $BaCl_2$ very possibly may be a combination of several effects, such as (1) a catalytic effect, at low concentrations; (2) a competitive effect with other metals; (3) a combination of Ba^{++} with the reaction product (citrate); or (4) a combination of Ba^{++} with a necessary component of the enzyme system (inorganic phosphate).

Fluoroacetate, at low concentrations (0.001 M) inhibited acetate oxidation (7, 8). Higher concentrations of fluoroacetate (0.01 M) had no effect on a number of isolated enzyme systems, including isocitrate dehydrogenase, α-ketoglutaric dehydrogenase, succinoxidase, malic oxidase, and oxaloacetic decarboxylase. Therefore, the inhibition of citrate utilization at 0.02 M fluoroacetate might possibly be a result of acetate inhibition, reflected through a series of reversible steps. This would be analogous to the effect of sodium fluoride on glucose utilization, or to the inhibitory effect of malonate on pyruvate oxidation. Aside from the inhibition of citrate utilization, the mechanism whereby

fluoroacetate increases citrate formation from oxaloacetate is obscure and will be further investigated in the near future.

SUMMARY

BaCl$_2$, MgCl$_2$ and sodium monofluoroacetate increase the formation of citrate from oxaloacetate by a rabbit kidney cortex homogenate. Mg^{++} is twice as effective as Ba^{++}. Ba^{++} seems to have more than one concentration optimum, and inhibits at higher concentrations.

MgCl$_2$ and sodium fluoroacetate are equally effective, over a wide range of concentrations. The other halogen acetates do not increase citrate formation from oxaloacetate.

The effects of BaCl$_2$ and MgCl$_2$ in increasing citrate formation can be accounted for by the inhibition of citrate utilization by the tissue, Mg^{++} being somewhat superior to Ba^{++} in this respect.

The slight inhibition of citrate utilization at higher concentrations of fluoroacetate can only account for about 60% of the increased citrate formation from oxaloacetate, in the presence of fluoroacetate.

The possible mechanisms of action of Ba^{++}, Mg^{++}, and fluoroacetate, in this system, are discussed.

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An Agar Plate Assay for Biotin

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INTRODUCTION

In 1889 Beijerinck (1) devised a gelatin plate technique for detecting soluble growth-promoting substances and proposed that it be called the auxanographic method. More recently Mager and Aschner (2) used this same principle for the detection of essential vitamins for encapsulated yeasts. In connection with studies in this laboratory on the metabolism of biotin, the need arose for a rapid method of assaying a large number of biotin samples. Since precise assay methods depending upon the diffusion of test substances through agar medium have been developed for the determination of antibiotic substances, such as penicillin, and have been widely used because of their speed and simplicity, it occurred to us that a similar type of assay might be developed for the determination of growth-promoting substances in general, and biotin in particular. In such an assay the growth response would be determined by measuring the diameter of the circle of growth, whereas in an antibiotic assay the response is determined by measuring the diameter of the circle of inhibition of growth.

Since this work was initiated, Bacharach (3) has called attention to the possibilities of the plate assay method for amino acids and vitamins of the B group and to several advantages inherent in this method, namely, the speed of the method and the greatly extended range of organisms available for use. While our manuscript was being prepared for publication, a note suggesting the use of the cylinder-plate method for the assay of biotin using a strain of *Saccharomyces cerevisiae* was published by Williams (4). At the same time, Price (5) briefly described a cup-plate method for the determination of riboflavin.

In the present studies on the use of solid media for the assay of biotin, it was found that the addition of solutions containing increasing concentrations of biotin to filter paper discs on a biotin-free but otherwise complete medium resulted in the production of graded zones of growth. A linear relationship was shown to exist between the diameter of the zone of growth and the logarithm of the dose over·at least a thousand-fold range of biotin concentrations. Contrary to the findings of both Bacharach and Price, the sensitivity of our agar diffusion method for biotin using filter paper discs was approximately the same as that encountered in assay methods making use of liquid media.

EXPERIMENTAL

Organisms

Two organisms were employed for the plate assays of biotin, namely, *Saccharomyces cerevisiae* (Fleischmann strain No. 139) and *Lactobacillus arabinosus* 17-5. The stock culture of yeast was transferred daily, except Sundays, upon wort agar (Difco) reinforced with an additional 1% agar. The inoculum was prepared by suspending several loopfuls of a 24-hour yeast culture in distilled water. The amount of yeast present in the suspension was determined by measuring the turbidity of the suspension and converting the turbidity readings to mg. of moist yeast/cc. by reference to a calibration curve. An inoculum equivalent to 1.2 mg. of moist yeast was used for each 100 cc. of agar medium.

The stock culture of *L. arabinosus* was carried in stabs of a dextrose yeast agar (6). On the day before use, the organism was inoculated into the basal medium plus 0.0002 γ biotin per tube (10 cc.). After incubation at 37°C. for 18–20 hours, the organisms were separated by centrifugation, washed once with saline, and resuspended in half the total original volume. The inoculum consisted of 2 cc. of this suspension/100 cc. of agar medium.

Medium for Assay

For the yeast assay, the medium of Snell, Eakin and Williams (7) was employed with the following additions per liter of medium: nicotinic acid 100 γ, p-aminobenzoic acid 20 γ, calcium pantothenate 40 γ, pyridoxine hydrochloride 70 γ, thiamine hydrochloride 20 γ, L-tryptophan 10 mg., DL-aspartic acid 200 mg., vitamin-free casein hydrolyzate (General Biochemicals) 5 cc. of a 10% solution, and sodium acetate 0.6 g. The resulting medium had a pH of about 4.5. To this was added 1.5 g. of agar (Difco)/100 cc. The medium was autoclaved, cooled to 45°C. and inoculated with the yeast suspension.

It has been the custom in this laboratory to free the sucrose of traces of biotin before using the sugar in a yeast assay for biotin. Better results were also obtained in the present assay if purified sucrose was used. The biotin was removed from the sucrose by a charcoal adsorption method (8). One kg. of sucrose was dissolved in 2 l.

of distilled water and the solution was stirred for 30 minutes at room temperature with 250 g. of Darco. The Darco was removed by filtration through a thin layer of diatomaceous earth (Filter Cel), and the sugar concentration was determined from the specific gravity of the solution. The filtrate was divided into portions containing 100 g. of sucrose, sterilized by autoclaving for a minimum length of time and stored until needed.

For the *L. arabinosus* assay, the medium of Snell and Wright (6) was used with the following exceptions: biotin was omitted from the medium and 500 γ nicotinic acid, 100 γ *p*-aminobenzoic acid, 100 γ calcium pantothenate, 150 γ pyridoxine hydrochloride, and 100 γ thiamine hydrochloride were added/l. of medium. Then agar (1.5 g./100 cc.) was added, the medium was autoclaved, cooled to 45°C., and inoculated with the *L. arabinosus* suspension.

Preparation of Plates

By means of a Brewer automatic pipette, the inoculated agar was distributed at a temperature of 45°C. in 16 cc. amounts into sterile petri dishes (100 mm. diam.) with unglazed porcelain tops (Coors). These plates were iced as soon as they had hardened. The plates can be stored at 5°C. for at least 1 week before use in an assay.

Assay Procedure

Dilutions of the biotin standard ranging from 1.0 to 0.001 γ/cc. were made from a 10 γ/cc. solution of natural biotin in distilled water. Filter paper discs (9, 10) (Schleicher and Schuell, No. 740-E) were placed on the surface of the agar and about 0.08 cc. of the biotin solutions was delivered onto them. For accurate results it is necessary to deliver the same volume of fluid to each pad, a tedious process if performed with an ordinary pipette. For this purpose a semiautomatic pipette (see below) was employed. This pipette, which delivers a constant volume each time it is emptied, has been used in this laboratory for several years in penicillin assays. The same pipette was used throughout the assay and rinsed between each sample.

Since the filter pads will rapidly absorb water from the moist agar, the biotin solutions were added to the pads as soon as possible after they had been placed upon the agar. In practice, from 5 to 10 pads could be placed on the agar at one time. The number of pads that were placed upon a plate depended on the size of the expected growth ring; for the lowest concentrations of biotin, 4 or 5 pads were applied to a plate, whereas in the 1.0 γ/cc. concentration range no more than 2 pads were applied. The plates were removed from the refrigerator in groups of 5–10 as needed and placed in the incubator as soon as all the solutions had been added. In both the *S. cerevisiae* assay and the *L. arabinosus* assay the plates were incubated at 30°C. for 18–20 hours. After incubation the diameters of the circles of growth about the filter pads were measured to the closest 0.5 mm. using a Penincillin Reader (Fischer). In the case of the *L. arabinosus* assay the area of the circle of growth was well defined, while in the yeast assay there was a small area of diffuse growth beyond the well defined edge of a band of heavy growth. The edge of the band of heavy growth was used for the measurement of the growth response. The standard curve was obtained by plotting the diameter of the circle of growth against the logarithm of the concentration of biotin.

Sterile Technique and Cleansing of Glassware

A minimum of sterile technique was necessary. Although the medium and petri dishes were sterilized before use, it was not necessary to sterilize the other glassware, filter paper discs, and solutions. In assaying for biotin in the past with other procedures we have found it necessary to cleanse thoroughly all glassware by immersion in a hot sulfuric acid bath. However, in the present procedure, although all glassware was washed thoroughly in alkaline soap solution, only the flasks for the medium and the containers for the biotin solutions were cleansed in the hot acid bath.

Semiautomatic Pipette

The design of the semiautomatic pipette is shown in Fig. 1. The pipette is constructed from pieces of pyrex glass tubing with outside diameters of 14 and 8 mm. and
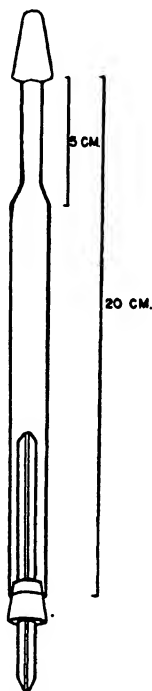
FIG. 1. Diagram of semiautomatic pipette.

a portion of a 0.1 cc. pipette. A 0.1 cc. pipette is heated in a flame and drawn out at a point where the end section would contain about 0.08 cc. The end section is severed from the rest of the pipette at this constriction. The constricted end of the pipette should come to a fairly sharp point, so that the overflowing liquid will be cleanly separated from the liquid remaining in the pipette. This section of the pipette is

inserted into the small half of a rubber stopper (size 00), which, in turn, is inserted into the wide end of the glass tube so that the constricted end of the pipette is enclosed in the chamber. A rubber bulb is attached to the narrow end of the glass tube.

In operation, the tip of the pipette is inserted into the solution contained in a test tube (20 × 150 mm.). Fluid is drawn up into the pipette by release of pressure on the rubber bulb; any excess fluid spills over the constricted end of the pipette into the closed chamber, thus giving an "automatic" volume control. In practice, when one has become skillful there is little spilling over. After the pipette is removed from the solution, the tip is wiped off and placed on the filter paper disc. The fluid contained within the pipette is discharged onto the filter paper disc by the application of pressure on the rubber bulb. To rinse the pipette between samples, fluid is forced up and down in the pipette as it is passed serially through 3 portions of distilled water. Finally the pipette is rinsed several times with the next sample before withdrawing a portion for assay. At the end of the assay, the whole apparatus can be disassembled for cleaning.

RESULTS AND DISCUSSION

The growth responses obtained for *S. cerevisiae* and *L. arabinosus* from 0.001 to 1.0 γ of biotin/cc. are shown in Figs. 2 and 3. Each point on the curve was determined by an average of 20 replicates.
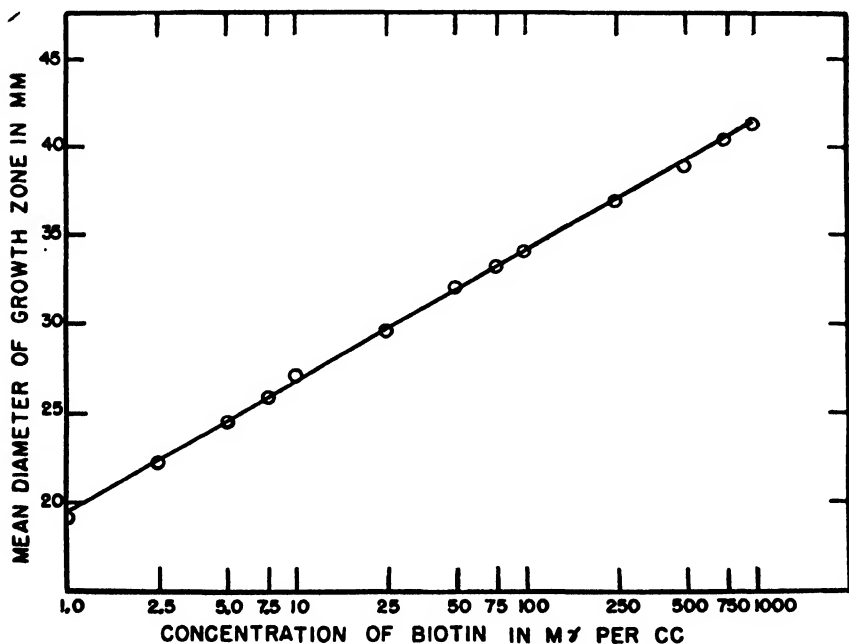
FIG. 2. Growth response of *S. cerevisiae* (Fleischmann strain No. 139) to biotin in agar plate assay.
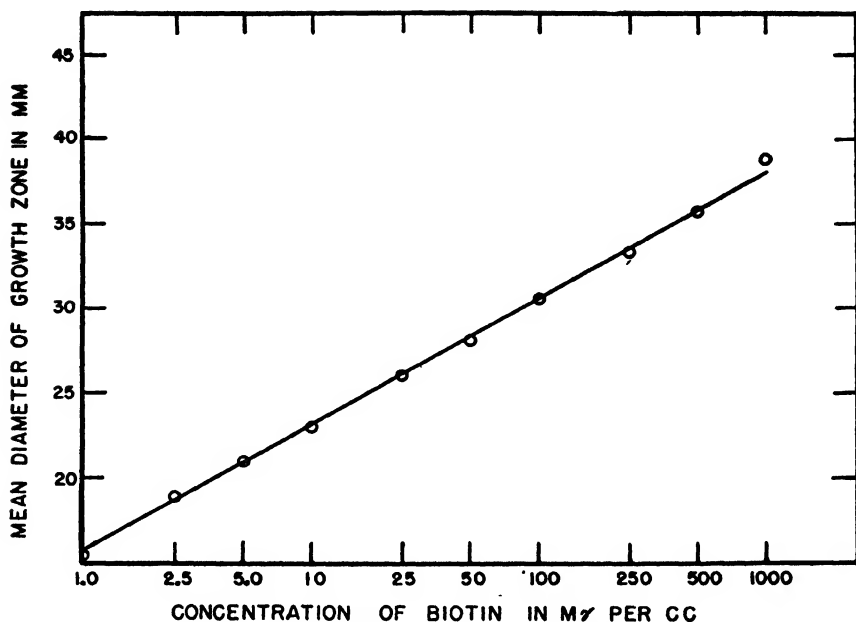
FIG. 3. Growth response of *L. arabinosus* 17-5 to biotin in agar plate assay.

The results show that the dose response curves of both organisms are linear over the range of 0.001–1.0 γ of biotin/cc. Thus, the linear response curve has a wider range of usefulness than that usually encountered in microbiological assays. An unselected portion of the results used in plotting the response curves are reproduced in Tables I and II. From a study of these figures one can obtain an estimate of the accuracy of the assay as well as the variation encountered between replicates on different plates. It should be noted that the sensitivity of this agar plate assay is about the same as that experienced in liquid media (7). Thus, a measurable zone of growth is obtained when as little as 0.00008 γ of biotin (0.08 cc. of a solution containing 0.001 γ/cc.) is applied to the filter paper disc.

The use of an agar plate assay for the detection of growth-promoting substances offers many possibilities. The application of this method should greatly extend the variety of organisms that may be used for assay work. Not every organism grows smoothly enough in liquid medium to be measured turbidimetrically, or, as also noted by Bacharach (3), produces enough acid to be measured acidimetrically; however, such organisms might be used to advantage in an agar plate

TABLE I

Variation of Growth Response of S. cerevisiae to Biotin

Plate no.	Zone diameters			
	0.025 γ biotin/cc.	0.050 γ biotin/cc.	0.075 γ biotin/cc.	0.100 γ biotin/cc.
	mm.	*mm.*	*mm.*	*mm.*
1	29.0	32.0	33.0	34.0
2	29.5	31.5	33.0	34.0
3	29.0	32.0	33.5	34.0
4	29.5	32.0	33.0	34.0
5	30.0	32.0	33.0	34.0
6	29.0	32.0	33.0	34.0
7	30.5	32.5	33.5	34.0
8	29.5	32.0	33.0	34.0
9	29.0	32.0	33.0	34.0
10	30.0	32.5	33.0	34.0

TABLE II

Variation of Growth Response of L. arabinosus to Biotin

Plate no.	Zone diameters			
	0.0025 γ biotin/cc.	0.005 γ biotin/cc.	0.010 γ biotin/cc.	0.025 γ biotin/cc.
	mm.	*mm.*	*mm.*	*mm.*
1	19.5	21.5	23.5	26.5
2	18.5	21.0	23.0	26.0
3	19.0	21.0	23.0	26.0
4	19.0	22.0	23.0	26.0
5	19.0	20.5	23.0	26.0
6	18.5	21.5	24.0	27.0
7	19.0	21.5	23.0	26.5
8	18.5	20.5	23.5	26.0
9	19.0	21.0	23.5	26.0
10	19.0	22.0	23.0	26.5

assay. It appears that the method might also be used for the determination of amino acids as well as vitamins. In preliminary experiments we have found that application of valine solutions of various concentrations to filter paper discs on a medium which was seeded with *Streptococcus faecalis* (ATCC No. 8043), and which was complete except for valine, gave graded growth responses.

A novel variation of the yeast agar plate assay for biotin has been developed in this laboratory. This variation has been used for the

detection of biotin and related vitamers on paper-strip chromatograms. The developed chromatograms were placed on a large plate containing the seeded basal agar. After the plates had been incubated, the areas of the strip containing biotin or biotin-like compounds were clearly shown by elliptical growth patterns.

It should also be pointed out that modifications of the agar plate assay might be used to advantage in the determination of antinutrilite compounds. The possibilities of this type of assay are being investigated.

SUMMARY

An agar plate paper disc assay for biotin, using both *Saccharomyces cerevisiae* Fleischmann strain No. 139 and *Lactobacillus arabinosus* 17-5, has been described. The linear dose response curve was found to have a wider range of usefulness (0.001-1.0 γ of biotin/cc.) than that usually encountered in microbiological assays. The method offers distinct advantages over assays for biotin employing liquid media, while retaining a comparable sensitivity. A considerable saving of time and effort is realized by a limited use of sterile technique and biotin-free glassware, and by the large number of samples that may be handled in any given period. There is an added convenience in that the seeded plates may be stored for at least a week before use.

In the discussion, various suggestions have been made for the extension of the use of solid media in microbiological assays.

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Biochemistry of Wound Healing. III. Total Lipide, Phospholipide, and Cholesterol Content of Skin and Repair Tissue of Skin Wounds

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INTRODUCTION

In a previous paper (1), we reported the water and protein content of repair tissue in normally healing skin wounds. It was found that water made up approximately 80% and protein 15% of the constituents of repair tissue, the remaining 5% presumably consisted mainly of lipides. In reviewing the literature, we have been unable to locate any data on the lipides of repair tissue of wounds. Since knowledge of changes in the basic composition of tissue is essential in a study of its metabolism, an investigation of lipide distribution in repair tissue was undertaken. The present paper is concerned with the total petroleum ether-soluble lipide, phospholipide (determined as phosphorus), and the total and free cholesterol content of skin and of the repair tissue of skin wounds of the albino rat at various stages of healing.

EXPERIMENTAL

Young, adult albino rats of both sexes averaging 235 ± 18 days of age and reared on a commercial dog food diet supplemented twice weekly with fresh lettuce and carrots were selected. The males averaged 430 ± 35 g.; the females 253 ± 19 g. The animals were distributed into groups as uniformly as possible with regard to age, weight and sex. The groups consisted of animals with standard wounds allowed to heal 3, 6, 9, 12, 18, and 131 days.

The wounds were produced under anesthesia in a manner previously described (2) with the exception that in this work, 3 wounds per animal were made to obtain sufficient material for analysis. One was placed on each shoulder and the third was placed medially and about 10 mm. caudad from the other two wounds. The sections removed at the time of wounding were analyzed to provide values for normal skin. The samples of scar tissue used for analysis 131 days after wounding were from single,

large wounds (about 25 mm. diam.). The healing time on these 131 day wounds, as judged by external appearance, was 30 days.

Measurements of the wounded areas were taken with calipers as previously described (2). At predetermined stages of healing, the animals were killed by etherization, the scab removed, and the unhealed portion measured. The repair tissue was then excised and used for analysis. At the 18th day and the 131st day, the scars from completely closed wounds were analyzed. When necessary, the repair tissue from several animals was pooled for analysis.

Analytical

The skin samples removed at wounding were combined and weighed, cut into small pieces and ground in a tissue homogenizer (3) with 5 ml. of 95% ethyl alcohol. The slurry was rinsed quantitatively into a 50 ml. Erlenmeyer flask using a total of 20 ml. of alcohol. The flask was heated on a steam bath for 15 mins. and the extract decanted. Extraction was then continued using ethyl alcohol-ethyl ether (3:1), followed by 2 more alcohol extractions. The combined extracts were evaporated at 50–60°C. almost to dryness under a stream of nitrogen. One ml. of 40% alcohol was added and the resulting mixture extracted 3 times with boiling petroleum ether.

The petroleum ether extract was decanted into a tared flask, evaporated to dryness and weighed. This represented the total petroleum ether-soluble lipide. The lipide was redissolved in petroleum ether and made to a suitable volume (10 ml.). Aliquots were taken for further analysis.

Total and free cholesterol were determined by a modification of the Schoenheimer and Sperry method (4). The concentration and amounts of reactants were increased to permit the convenient use of a 10 mm. absorption cell in a Beckman spectrophotometer. Samples contained 0.25–0.75 mg. cholesterol. Precipitation of the cholesterol digitonide was made with 0.4% digitonin solution. All quantities of the saponification and precipitation reagents were tripled. The color development was carried out on the cholesterol digitonide with double the amount of reagents described in the original method (4). With the above modification, recoveries of 0.25 mg. cholesterol added to skin extract was 104%, 0.50 mg. added gave 99% recovery.

Phospholipide was determined as lipide phosphorus by a modification of the magnesium nitrate ashing procedure of Roepke (5) which permitted the determination of the phosphorus spectrophotometrically by the molybdenum blue method of Holman (6). Since many modifications were necessary, the method used is described in detail.

A measured amount of petroleum ether extract containing 5–15 γ of phosphorus was pipetted into a 4 ml. porcelain crucible and evaporated to dryness with the aid of an air jet and a hot plate. One-half ml. of 25% $Mg(NO_3)_2 \cdot 6H_2O$ in 95% ethyl alcohol was added to each crucible and evaporated to a thick yellow liquid. Heat was increased until a portion of the ash became white. The crucible was placed in a muffle furnace at 700°C. until fuming ceased. When any organic matter remained, a few drops of conc. HNO_3 were added and the heating repeated. After all the carbon had been oxidized, the crucible was heated for 6–10 hours in the muffle furnace. The crucibles were cooled and 1 ml. of 1.2 N HCl and 1 drop of 1% alcoholic phenolphthalein were added. The crucibles were then placed on a steam bath and heated

one hour with occasional stirring. All the residue dissolved in this time. The crucibles were cooled and rinsed with 4 ml. of distilled water into lipless test tubes graduated at 5 ml. and 10 ml. The contents were neutralized to phenolphthalein with 0.5 N NaOH and made to 5 ml. with distilled water.

To the tubes 1 ml. of 10 N H_2SO_4, 1 ml. of 2.5% $(NH_4)_2MoO_4$, and 1 ml. of 20% KI containing 0.5% Na_2CO_3 were added in the order named, with mixing after each addition. The tubes were covered and placed in boiling water for 15 mins., cooled, and titrated with freshly prepared 0.5% Na_2SO_3 solution to a pure blue color. An excess of 0.2 ml. of sulfite solution was added, the contents diluted to 10 ml., and read on the Beckman spectrophotometer at 825 mμ against a water blank. A reagent blank was also run to correct for any trace of phosphorus found in the reagents. This was usually less than 10% of the total value. A concentration of 1 γ phosphorus/ ml. has an optical density of 0.80 at 825 mμ. With this method, 98% of 10 γ of added phosphorus was recovered from a skin lipide extract. Phospholipide was calculated by multiplying the phosphorus values by a factor of 25.

At predetermined stages of healing, the repair tissue was removed and treated in the same manner as skin.

RESULTS

The lipide values for skin, calculated on the basis of fresh weight, and tabulated according to sex, are to be found in Table I. Total

TABLE I
Lipide Content of Rat Skin—Fresh Weight Basis

Sex	No. of rats	Total lipide	Phospholipide (P×25)	Total cholesterol	Free cholesterol	Free/total cholesterol	Phospholipide/total cholesterol
		Per cent	*Per cent*	*Per cent*	*Per cent*		
♂ Skin	46	12.5±3.7	0.403±0.046	0.354±0.053	0.118±0.009	0.349±0.05	1.16±0.21
♀	17	22.0±2.3	0.416±0.029	0.248±0.020	0.132±0.010	0.500±0.05	1.68±0.19

petroleum ether-soluble lipide was found to be 75% greater in female skin than in male skin. Haldi *et al.* (7) found the total fatty acid content of female rat skin to be 82% greater than that of male rat skin. We found no significant sex difference in phospholipide and free cholesterol, but total cholesterol was higher by 42% in male skin than in female skin. The female skin has a higher free cholesterol to total cholesterol ratio, as well as a higher phospholipide to cholesterol ratio than male skin.

The lipide distribution in repair tissue on the basis of fresh weight is shown in Table II, tabulated according to sex and stage of healing.

TABLE II

Lipide Content of Repair Tissue—Fresh Weight Basis

Stage of healing Days	Sex	No. of rats	No. of wounds	Total lipide No. anal.	Per cent	Phospholipide P×25 No. anal.	Per cent	Total cholesterol No. anal.	Per cent	Free cholesterol No. anal.	Per cent	Free/total cholesterol No. anal.	Ratio	Phospholipide/total cholesterol No. anal.	Ratio
3	♂	6	18	5	4.73±1.85	5	0.58 ±0.053	5	0.181±0.009	5	0.127±0.004	5	0.687±0.058	5	3.22±0.26
	♀	2	6	1	3.86	1	0.618	1	0.168	1	0.116	1	0.690	1	3.68
6	♂	12	36	4	2.89±0.58	4	0.848±0.017	4	0.208±0.011	4	0.149±0.011	4	0.717±0.08	4	4.08±0.18
9	♂	4	12	1	3.26	1	0.955	1	0.270					1	3.54
	♀	4	11	1	2.72	1	0.890	1	0.252					1	3.54
12	♂	15	45	2	2.93±0.55	2	0.872±0.018	2	0.247±0.010					2	3.59±0.26
18	♂	7	18	2	3.53±0.81	2	0.756±0.017	2	0.352±0	1	0.192	1	0.607	2	2.15±0.05
	♀	7	21	1	4.22	1	0.774	1	0.316					1	2.45
131	♂	2	6	2	3.58±0.55	2	0.470±0.073	2	0.486±0.120	2	0.098±0.008	2	0.211±0.032	2	0.99±0.09
	♀	4	4	2	5.14±1.21	2	0.308±0.098	2	0.503±0.207	1	0.091	1	0.308	2	0.69±0.02

The total lipide content of repair tissue tended to remain the same throughout healing, and was considerably lower in repair tissue than in skin. No significant differences in the total lipide of repair tissue due to sex or stage of healing were noted.

The phospholipide content of repair tissue was consistently higher than that of intact skin. It increased during healing to a maximum and then declined. This relationship is shown in Fig. 1. No sex differences were noted in the phospholipide content of repair tissue.
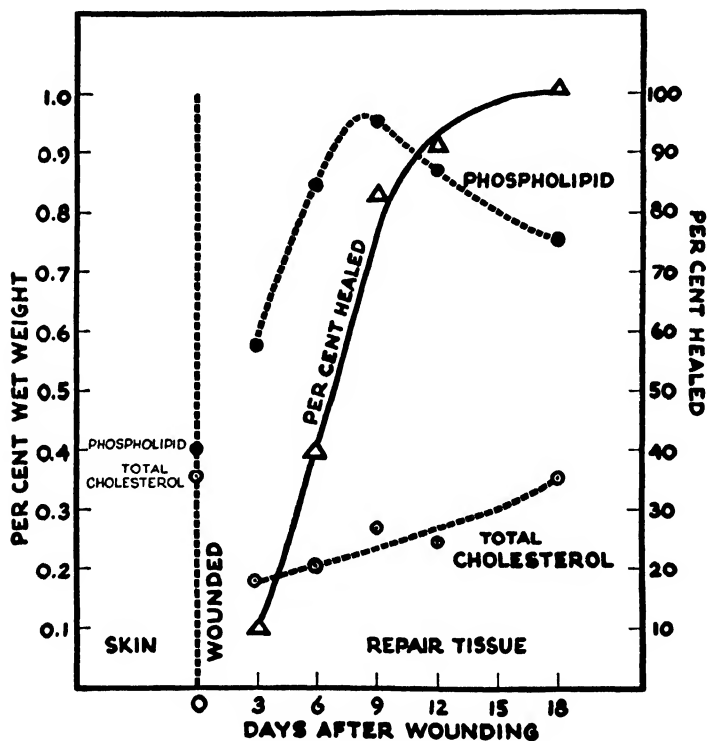
FIG. 1. Relationship between phospholipide and total cholesterol content of repair tissue and rate of healing.

The total cholesterol content of repair tissue is lower than in normal skin at the early stages of healing, but gradually rises (Fig. 1). At 18 days, it has risen to a value equal to that of skin. Total cholesterol in scar tissue (131 days) appears to be higher than in normal skin (Table II). No sex differences were observed.

Too few analyses of free cholesterol in repair tissue were made to establish its concentration at the various stages of healing. From the data, it would seem that it does not vary much during healing and that the values lie close to those found for skin.

The ratio of phospholipide to total cholesterol in tissues is considered an indication of metabolic activity. This ratio is higher for repair tissue at all stages of healing than for normal skin.

DISCUSSION

In addition to confirming the work of Haldi *et al.* (7) on the marked sex difference in the lipide content of rat skin, we found that, within the total lipide fraction itself, a significant sex difference appeared.

TABLE III

Phospholipide and Total Cholesterol Content of Skin and
Repair Tissue on Basis of Total Lipide

Healing stage		Sex	Phospholipide (P×25)	Total cholesterol
Skin	*Days* —	♂	*Per cent* 3.23	*Per cent* 2.83
	—	♀	1.89	1.13
Repair tissue	3	♂	12.3	3.82
		♀	16.0	4.35
	6	♂	29.3	7.20
	9	♂	29.3	8.26
		♀	32.8	9.26
	12	♂	29.8	8.46
	18	♂	21.4	10.0
		♀	18.4	7.5
	131	♂	13.1	13.6
		♀	6.0	9.8

The total cholesterol content of skin is 43% higher in males than in females. Since there is no sex difference in phospholipide content, it is evident that the phospholipide to total cholesterol ratio is higher in female skin than in male skin. Free cholesterol was found to be lower (11%) in male skin than in female skin. The free to total cholesterol ratio is higher in female skin than in male skin.

It is of interest to note that the sex difference, so apparent in skin lipides, seems to be lacking in repair tissue.

Although the 18 day wounds are healed, as far as can be judged from appearances, their composition indicates that healing processes are still continuing. The phospholipide content of 131 day scar tissue is similar to normal skin, cholesterol content is slightly higher, but the total lipide content remains decidedly lower.

Comparison of Tables I and II reveals that skin is lower than repair tissue in metabolically active fat, *i.e.*, phospholipide. Since the total amount of fat is high, skin serves largely as a fat storage organ. The lipide of repair tissue, although low in total amount, is primarily of a metabolically active nature. This point is emphasized when the values for skin and repair tissue are calculated on the basis of per cent of total lipide (Table III). Phospholipide is shown to make up about 30% of the lipide of repair tissue at the stage of most rapid healing as compared to roughly 3% for skin. The values for cholesterol, as calculated in Table III, are of interest in indicating that cholesterol makes up a much larger portion of the lipide of repair tissue, than of skin. Further investigation on the role of cholesterol as a structural element in scar formation would be desirable.

SUMMARY

1. Total petroleum ether-soluble lipide, phospholipide, total cholesterol, and free cholesterol have been determined in skin and repair tissue of skin wounds of rats at various periods of healing.

2. Total lipide makes up 22.0% of female skin and 12.5% of male skin. Total cholesterol is higher for male skin (0.354%) than for female skin (0.248%).

3. Total lipide makes up about 3–5% of repair tissue and does not vary significantly with sex or stage of healing.

4. Phospholipide of repair tissue increases during healing to a maximum value about double that of skin and then declines.

5. The total cholesterol content of 3 day repair tissue is about one-half that of skin, but slowly increases, reaching a value higher than that for normal skin in the scar at 131 days.

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Biochemistry of Wound Healing. IV. Oxygen Uptake of Healing Tissue of Skin Wounds

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INTRODUCTION

In recent years the metabolism of a variety of tissues has been studied, using manometric methods. In general, more active or rapidly growing tissues, such as sarcoma, embryonic and liver tissues, have a higher rate of metabolism than less active tissues, such as skin or muscle. Of the many tissues studied the repair tissue of actively healing wounds has not been thoroughly investigated.

Neuhaus (1), working with rats, found that the ratio of aerobic glycolysis to respiration was almost as high for granulation tissue as for rat sarcoma. Ryvkina and Striganova (2) found that, in the course of healing of skin wounds of rats, the gaseous metabolism increased, the maximum occurring at the time of the greatest growth of granulating tissue. Others, working with different types of wounds, von Gaza and Gissel (3) using a cleft type of human skin wound, von Gaza, Gerlach and Gissel (4) using the Achilles tendon of dog, and Fardon *et al.* (5) studying X-ray burns on mice, all found essentially the same results—a decrease in oxygen uptake immediately after wounding, followed by an increase during repair, with a subsequent decline toward normal as healing neared completion.

Since the above workers were dealing with differing types of wounds, or differing species of animals, it was considered of value to repeat and extend these observations on the standard type of skin wound with which our research has been concerned. In a previous paper (6) we mentioned that preliminary experiments with skin wounds of rats indicated that the Q_{O_2} of repair tissue was higher than that of skin. In this paper we present the data from a more complete investigation of the oxygen uptake of repair tissue at various stages of healing. Normal adult skin and the skin of 3 day old rats were also studied for comparative purposes.

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EXPERIMENTAL

Circular skin wounds about 1 cm. in diameter were produced on both shoulders of healthy rats of 121–136 days of age by the method described in our previous paper (6). At predetermined intervals of 3, 6, 9, 12, and 18 days after wounding, the right hip of selected animals was dry shaved and the animals killed by decapitation. Sections of skin were removed from the shaved area for determination because of the possibility that the wound may have elaborated a substance stimulatory to skin respiration (2). The skin was weighed and kept at 10°C. in a closed chamber until sliced (7). Then the two wounds on each animal were measured after removal of the scabs, the repair tissue carefully dissected out, combined, weighed, and kept at 10°C. until sliced.

The tissues were moistened with Ringer's solution, sliced into strips 0.5–1.0 mm. thick and placed in 15 ml. Warburg flasks. The medium consisted of 3 ml. of Ringer's glucose-phosphate (0.2% glucose and 0.01 M phosphate, final pH 7.4) with 0.3 ml. of 15% KOH in the center well. The bath was maintained at 38°C. All experiments were conducted in an atmosphere of oxygen using the direct method of Warburg (8). After a 15 minute equilibration period, the system was closed and the uptake measured at the end of 1 hour. Oxygen uptake was calculated on the basis of mm.^{3}/mg. fresh tissue/hr.[2]

About 30 minutes elapsed from the time of removing the tissues from the animals until the placing of the flasks in the bath. Skin was difficult to slice because of its extreme toughness. A minimum amount of Ringer's solution was used during its slicing to prevent excessive swelling. Repair tissue was of a gelatinous structure, especially at the 3 day stage, so that slicing was difficult. The skin of 3 day old rats offered no difficulties in preparation.

Determinations on skin were made in duplicate, as sufficient sample was available. The sample size varied from 0.1–0.4 g. Difficulty was encountered in obtaining sufficient repair tissue in the later stages. Three and six day wounds offered no difficulty, but for 9, 12, and 18 day wounds it was necessary to composite tissue from rats at the same stage of healing. The sample available was in some cases as low as 0.05 g., but we have found that the Q'_{O_2} of skin or repair tissue was not influenced by sample sizes in the range used. In certain preliminary experiments which were conducted over a 2 hour period, we observed that the respiration decreased 5% for skin and 10–15% for repair tissue during the second hour.

RESULTS

Data for the oxygen uptake of adult rat skin and of repair tissue at various stages of healing are presented in Table I. Since we were dealing with such small samples of tissue, moisture determinations were considered to be subject to too great errors to be useful. The Q'_{O_2} (fresh weight) values for skin and repair tissue have been calculated to Q_{O_2} (dry weight) values by the use of the average figures for moisture which we determined in a previous experiment (11) on similarly treated

[2] For mm.^{3}/mg. fresh tissue/hr. we use the symbol Q'_{O_2}.

TABLE I

Oxygen Uptake of Normal Skin and Of Repair Tissue
at Different Stages of Healing

Stage of healing	No. rats	Skin Q'_{O_2}	Repair tissue Q'_{O_2}	Skin QO_2	Repair tissue QO_2	Moisture[a] repair tissue
Days						*Per cent*
3	6	0.19 ± 0.04	0.33 ± 0.04	0.45	1.93	82.9
6	10	0.22 ± 0.04	0.46 ± 0.09	0.52	2.47	81.4
9	10	0.24 ± 0.07	0.41 ± 0.06	0.57	2.17	81.1
12	14	0.22 ± 0.04	0.31 ± 0.05	0.52	1.73	82.1
18	16	0.20 ± 0.02	0.35 ± 0.10	0.47	1.38	74.7

[a] See Ref. (11). Skin moisture for animals of similar age was found to be 57.8%.

animals. Inspection of Table I reveals that oxygen uptake by normal skin is apparently not appreciably influenced by the stage of healing of a wound present at another site. Repair tissue has a higher oxygen uptake than normal skin and the oxygen uptake varies with the stage of healing, the highest Q'_{O_2} or Q_{O_2} occurring at the stages of most rapid healing, and then declining. This relationship is readily demonstrated in Fig. 1. It is to be noted that, even at the 18th day, the oxygen uptake of the repair tissue was much higher than that of normal skin. Undoubtedly, healing was still in progress at this time, even though closure of the wound was complete. It is probable that if we had carried our experiments beyond this time the Q_{O_2} of the repair tissue would decline to that of normal skin if these wounds follow the same trend as those of Fardon *et al.* (5) and von Gaza *et al.* (4).

Table II shows the data for skin from the 3 day old rat and for male and female adult skin of all animals studied. As can be seen, the Q'_{O_2} (*i.e.*, fresh weight basis) for males is practically the same as for females,

TABLE II

Oxygen Uptake of Normal Rat Skin

Skin	Sex	No. of rats	Q'_{O_2}	QO_2	QO_{2p}[a]
			Fresh wt.	*Dry wt.*	
Adult	Male	30	0.22 ± 0.04	0.54 ± 0.11	0.90 ± 0.16
	Female	26	0.21 ± 0.04	0.45 ± 0.08	0.93 ± 0.18
3 day rat		4	0.81 ± 0.03		.

[a] Mm.³ oxygen/mg. protein/hr.
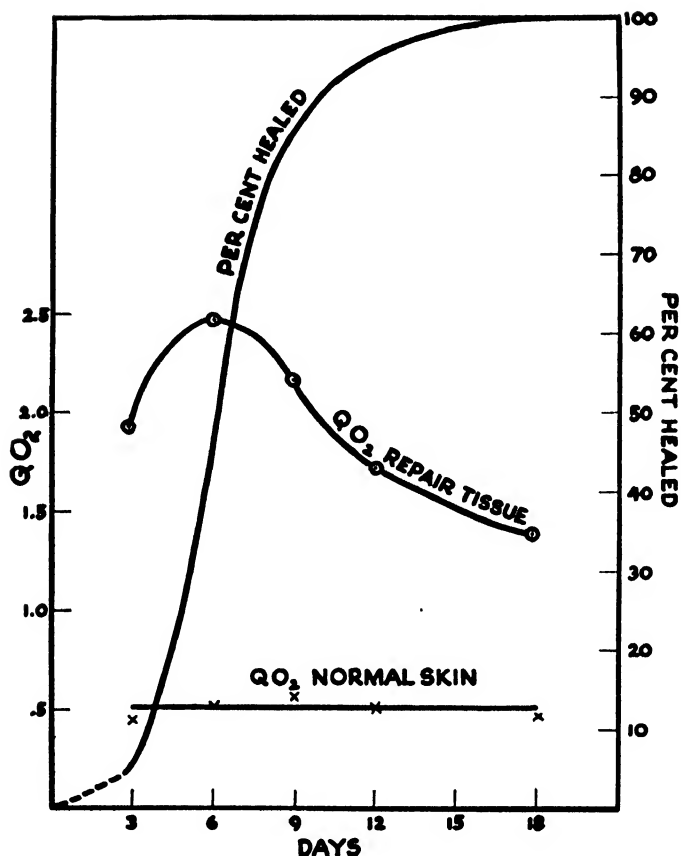
Fig. 1. Relationship between oxygen uptake of repair tissue and rate of healing.

but, when the value is calculated on the dry weight basis (Q_{O_2}), using the average figures for water found by us in previous work (11) (60% for males and 54% for females), males have a Q_{O_2} of 0.54 ± 0.11 and females a Q_{O_2} of 0.45 ± 0.08. The skin of the three day old rat shows a very high Q'_{O_2} as compared to adult skin or to repair tissue.

Discussion

· Cook *et al.* (9) found a Q_{O_2} of 1.0–1.15 for the skin of 2 males and 0.45–0.65 for that of 2 females. Although our data show that male skin

tends to have a higher Q_{O_2} than female skin, the difference cannot be considered significant. If we calculate the oxygen uptake on the basis of protein content, using the average protein values of 24.6% for males and 22.6% for females found by us in previous work (11), to eliminate the factor of larger amounts of inactive fatty tissue in female skin (10), we obtain values of 0.90 ± 0.16 and 0.93 ± 0.18 mm.³/mg. protein/hr. for males and females, respectively. Thus, when our data are calculated on the basis of active tissue, we find no significant sex difference in the rate of oxygen uptake by the skin of male and female rats.

Fig. 1 shows the relationship between the oxygen uptake of skin, oxygen uptake of repair tissue, and the per cent of healing. Although a slight increase in the oxygen uptake of skin appears at a stage of most rapid healing, this increase is not significant. The oxygen uptake of repair tissue increases markedly at the stages of most rapid healing confirming the work of Ryvkina and Striganova (2). In the type of wound studied in our experiments, the most rapid healing is taking place between the 6th and 9th day stages, and the wound is completely healed by the 18th day, as judged by external appearances. However, as judged by either chemical composition (11) or by metabolic rate, healing processes are still continuing.

Since measurement of oxygen uptake in a tissue represents a summation of all the oxidative metabolism taking place in that tissue, it is of interest to note the relation of oxygen uptake to the healing process, as evidenced by the curve for Q_{O_2} in Fig. 1, i.e., rising to a maximum at the stage of most rapid healing. We believe it is of importance that this type of curve was also found for thiamine concentration during wound healing (6) and for phospholipide (12). These tissue constituents should be considered as metabolically active substances. In contrast, we find a different type of curve, i.e., a gradual increase throughout the entire healing process, for tissue constituents which may be considered structural, such as protein (11) and probably cholesterol (12).

SUMMARY

1. The oxygen uptake for repair tissue of normally healing wounds, adult rat skin and skin of 3 day old rat has been determined.

2. Repair tissue has a higher oxygen uptake than adult skin. The maximum oxygen uptake of repair tissue has been found to occur at the stage of most rapid healing.

3. The oxygen uptake of the actively growing skin of the 3 day old rat is higher than that of the skin of the adult rat or of repair tissue of skin wounds.

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Nutritional Studies on Subtilin Formation
by *Bacillus subtilis*

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INTRODUCTION

This paper deals with the general nutritional factors conducive to the formation of relatively high levels of subtilin on simple synthetic media in shallow-layer stationary cultures, and with applications of these findings to the practical production of the antibiotic. Detailed investigations of the mineral requirements are reported elsewhere (1, 2).

Subtilin production has been studied in surface cultures on asparagus butt juice media (3), synthetic media (4), and recently on a variety of

TABLE I

Growth and Antibiotic Activities on Several Media

Culture medium	Time of incubation (hrs.)										
	20	24	29		40		48		72	
	Activity	Activity	Dry wt.	Activity	Dry wt.	Activity	Dry wt.	Activity	Dry wt.	Activity
	mg./l.	*mg./l.*	*mg.*	*mg./l.*	*mg.*	*mg./l.*	*mg.*	*mg./l.*	*mg.*	*mg./l.*
Asparagus juice[a]	430	690	850	530	820	560				
Beet molasses[b]		∼20		∼35	990	200	1480	530	2130	1330
Base A[c]					300	330	420	530	480	620
Base C[c]					110	120	160	210	550	960

[a] 150 g. of asparagus butt press juice/l. (to give 10% solids, 6.6% sugar).

[b] Beet molasses to give 20% solids and 15% sugar + 0.8% $(NH_4)_2HPO_4$ and 50 p.p.m. Mn.

[c] See Table II.

media in surface and submerged cultures (5, 6). The asparagus butt juice and beet molasses media gave higher antibiotic activities than media previously studied, but the former induced more rapid formation of antibiotic and easier chemical purification, while the latter gave twice the final antibiotic activities (Table I). Initial attempts to combine these two media, or to supplement one medium with chemically prepared fractions of the other, were unsuccessful and it appeared necessary to make more fundamental studies of the nutrition of the organism.

MATERIALS AND METHODS

The culture used in these studies was the non-adherent stock previously described (5). For use the culture was transferred to, and then subcultured once on, the particular test medium under study. The subculture was dispersed by shaking manually, and 0.5 ml. was used as an inoculum for each flask of experimental medium.

Experiments were conducted in 50 ml. volumes of media in 250-ml. Erlenmeyer flasks. All vessels and pipettes were of pyrex glass and were cleaned with dichromate cleaning solution and well rinsed with redistilled water prior to use. Pipettes were sterilized unwrapped in pyrex glass cases. Redistilled water was used throughout. Media were constituted double strength, the pH was adjusted to 6.9–7.0, and 25 ml. was added to each flask. The neutralized supplement under test, and water to give a final volume of 50 ml., were added. The flasks were plugged with cotton, sterilized at 120°C. and 15 lbs. pressure for 15 minutes, inoculated, and incubated at 35°C. for 68–72 hours unless otherwise indicated.

The asparagus press juice and beet molasses media were prepared as previously described (5). The formulae for the 3 synthetic media employed for most of these studies are listed in Table II. All ingredients of synthetic media were of reagent quality unless otherwise indicated.

Antibiotic Assays and Analyses

Antibiotic assays were performed by a bacteriostatic method (7) with *Micrococcus conglomeratus* as the test organism. Results of antibiotic assays were expressed in mg./l. of medium as determined against the arbitrary subtilin standard[2] (a partially purified sample, Lot L1263) used in the Western Regional Research Laboratory (7).

The dry weights of pellicles were employed as a quantitative measurement of the extent of growth and were obtained by centrifuging the pellicles from the medium, washing the pellicles once with water, and drying at 50°–60°C. for 24–48 hours. The dried pellicles were also employed for the determinations of N, P, and S. N was determined by Kjeldahl analysis; P by the Allen method; and S by the Parr bomb method. Residual sucrose in the media was determined by the Schaeffer-Hartmann method after removing the pellicles by centrifugation and inverting the carbohydrate.

[2] Samples of purified subtilin now available in this laboratory are approximately twice as active as the standard employed in this work (9).

TABLE II

Composition of Base Media

Ingredient	Amount added per liter of medium		
	Base A	Base B	Base C
Sucrose	100 g.	100 g.	100 g.
Na_2SO_4	4.0 g.	4.0 g.	4.0 g.
NaCl	0.3 g.	0.3 g.	0.3 g.
$(NH_4)_2HPO_4$	8.0 g.	—	—
$Na_2HPO_4 \cdot 12H_2O$	—	*f*	11.6 g.
Diammonium citrate	—	—	24.2 g.
Citric acid	0–5 g.[a]	6.0 g.	—
Asparagin[b]	2.0 g.	8.0 g.	—
Glutamic acid[c]	2.0 g.	8.0 g.	—
Salt mixture A[d]	100 ml.	100 ml.	—
Salt mixture B[e]	—	—	100 ml.
Total N, per cent	0.23	0.23	0.30
Organic N, per cent	0.06	0.23	0.00
Inorganic N, per cent	0.17	0.00	0.30
pH at final harvest	5.8–6.0	6.6–6.9	6.6–7.0
Time of incubation (hrs.)	60–72	48–60	60–96

[a] Unless otherwise noted, 2.0 g. of citric acid were added/l. of medium.

[b] Various lots of asparagin were employed. All lots were practical or "purified for bacteriological use." No differences were noted among them.

[c] Both technical L-glutamic acid and synthetic DL-glutamic acid were used with no significant differences noted.

[d] Salt Mixture A contained the following salts/l.: KCl, 3.81 g.; $MgCl_2 \cdot 6H_2O$, 4.18 g.; $ZnCl_2$, 0.104 g.; $FeCl_3 \cdot 6H_2O$, 0.245 g.; $MnCl_2 \cdot 4H_2O$, 0.181 g. When added to the base media in the amount indicated, it gave in p.p.m.: K, 200; Mg, 50; Zn, 5.0; Fe, 5.0; and Mn 5.0.

[e] Salt Mixture B was similar to Salt Mixture A with the exception that it contained double the amount of KCl, thereby giving a concentration of K of 400 p.p.m. in the medium.

[f] Base B was used to determine the requirement for P for growth and antibiotic production (Fig. 2).

RESULTS

Preliminary investigations showed that the nutritional requirements for the production of high levels of antibiotic activity were simple and were restricted to: (a) an appropriate source of carbohydrate, (b) in-

organic sources of S, P, and N, and (c) properly balanced amounts of mineral salts (2). All three of the media in Table II gave moderate to high yields of antibiotic activity. It was possible to subculture serially on Base Media A and C with no apparent diminution in the antibiotic activities obtained. Base Medium A gave the least variable results.

Carbohydrate Requirement

When glucose, fructose, galactose, xylose, mannitol, glycerol, or maltose was substituted for sucrose in Base Medium A, good growth, as determined visually, was obtained. Antibiotic activities (mg./l.) in this experiment were: sucrose, 630; glycerol, 620; glucose, 430; maltose, 310; and all others <200. The minimum requirement for sucrose was approximately 5–7% on Base Medium A. One per cent or less of sucrose resulted in abnormal (slimy) and light growth; 15% was
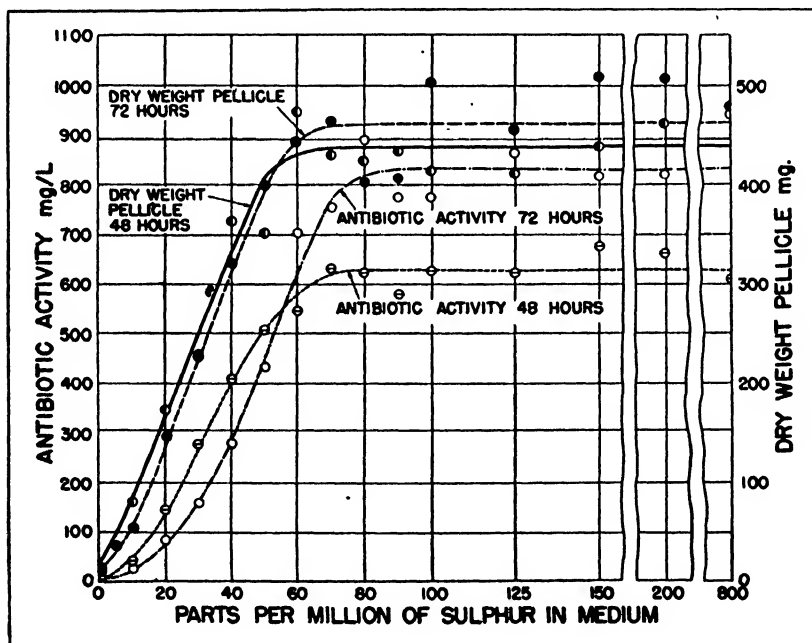
FIG. 1. Sulfur requirement for growth and antibiotic activity. This experiment was performed on Base Medium A with S omitted. The inoculum was grown on Base Medium A with S content reduced to 100 p.p.m. The antibiotic activities and dry weights of pellicles were determined on replicate sets of flasks after 48 and 72 hours of incubation. Graded amounts of S were added as Na_2SO_4.

tolerated; 20%, and especially 30%, retarded but did not inhibit growth.

Determinations of residual sugar on Base Media A and C after 72 and 120 hours of incubation gave the following results: on Base Medium A, 36 g./l. after 72 hours, and 35 g./l. after 120 hours; on Base Medium C, 14 g./l. after 72 hours, and 3.9 g./l. after 120 hours.

Sulfur Requirement

The minimum requirement for S was approximately the same for both growth and antibiotic activity (Fig. 1). For the 72-hour harvest, however, the requirement for antibiotic activity was possibly slightly greater than for growth.[3] Calculated on the basis of the production of 500 mg. dry weight of pellicle/50 ml. of culture, the minimum requirement for growth was approximately 80 p.p.m.

Analyses of pellicles for S did not reveal any significant differences between pellicles produced by cultures containing suboptimal or adequate amounts of S or by cultures harvested at 48 or 72 hours. The S contents varied from 0.35 to 0.44% and only about 60% of the added S was recovered in pellicles from cultures grown in the presence of suboptimal amounts of S.

Phosphorus Requirement

The quantitative requirement for P as Na_2HPO_4 was determined on Base B (Fig. 2). There was a definite optimum range for maximum antibiotic activity between 100 and 200 p.p.m. of P, but no such range was found for growth. The final pH's of the cultures in these experiments were 6.5–6.8 with no correlation between the final pH and anti-

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[3] The stability of the antibiotic activity in cultures was greatly dependent upon the medium. On asparagus press juice antibiotic activity usually decreased 25–50% when cultures were incubated for 24–48 hours after maximum antibiotic activities were obtained. In contrast, the activity of cultures on Base Media A and C remained essentially the same with prolonged periods of incubation. In an experiment on Base Medium A replicate flasks of cultures were harvested after 3 and 18 days of incubation. The flasks harvested after the 3-day period contained the usual maximum activity attained on this medium, 700–750 mg./l. The flasks harvested after the 18-day period contained 600–700 mg./l. However, under some suboptimal nutritional conditions appreciable losses were encountered in short periods. Examples of such losses were the results obtained with suboptimal levels of S (Fig. 1), in which activities at 72 hours were 30–45% lower than at 48 hours.
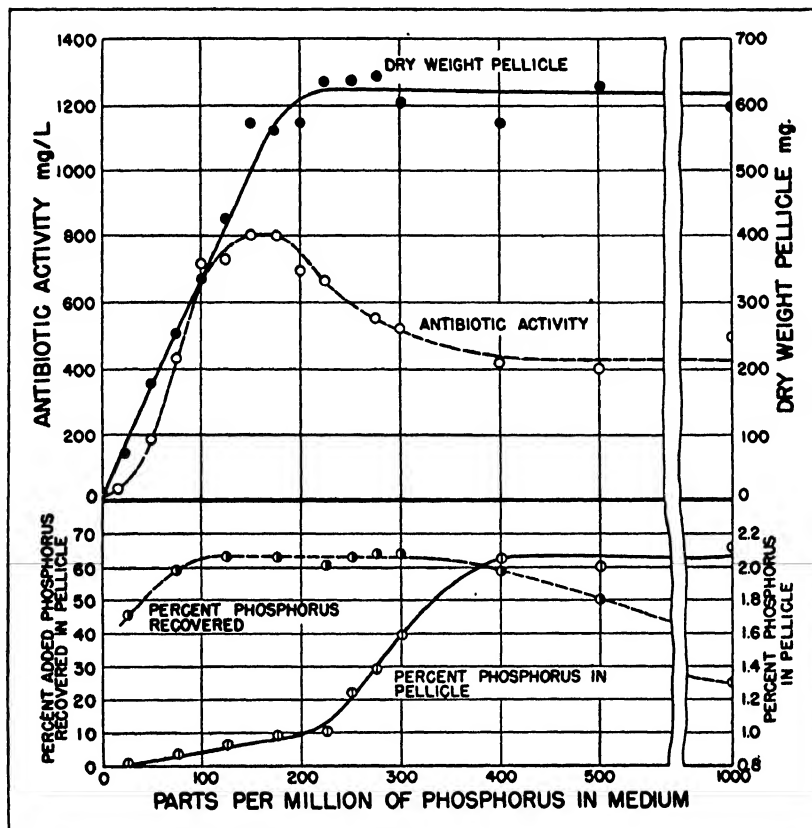
FIG. 2. Phosphorus requirement for growth and antibiotic activity. This experiment was performed on Base Medium B. The inoculum was grown on Base Medium B with 100 p.p.m. added P. Graded amounts of P were added as $Na_2HPO_4 \cdot 12H_2O$.

biotic activity noted. In contrast to these results on Base Medium B, were those obtained on Base Media A and C, in which high antibiotic activities were obtained with high P concentrations.

Nitrogen Requirement

The response to graded additions of N was determined by varying the NH_3 content in Base C (Fig. 3). A maximum of 54% of the added N was recovered, even with suboptimal levels of N. In experiments with the addition of an asparagus juice ash in which very heavy growth
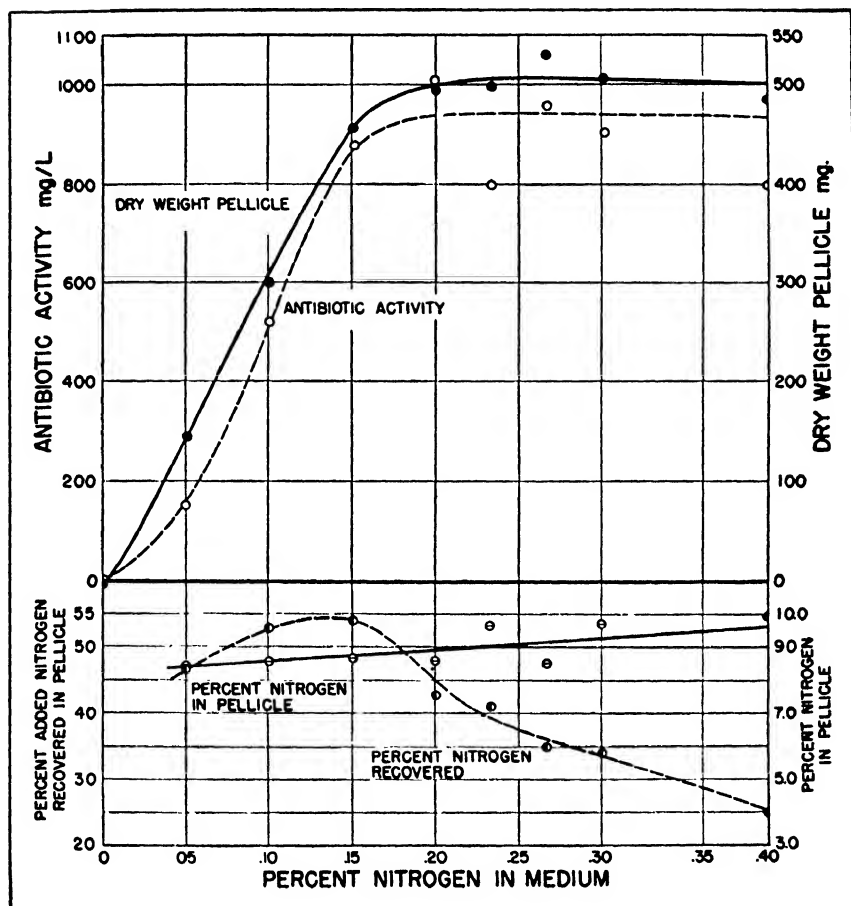
FIG. 3. Nitrogen requirement for growth and antibiotic activity. This experiment was performed on Base Medium C with N omitted. Graded amounts of N were added as diammonium citrate. The concentration of citrate ion was kept constant by the addition of citric acid.

occurred (2), as much as 82% of the added N was recovered in the pellicles.

A variety of N sources could apparently be utilized for growth and possibly for antibiotic formation. Good growth was obtained on glutamic and aspartic acids, NH_3, and KNO_3 or $NaNO_3$. In another experiment, antibiotic activities were 720 mg./l. with $NaNO_3$ and 760

mg./l. with KNO_3. Betaine, urea, and urethane did not support growth and $(NH_4)_2SO_4$ was unsatisfactory, probably because of acidity produced when the NH_3 was utilized.

Effects of Organic Supplements

While supplementations of Base Media A and C indicated that organic materials might increase the rate of growth with a consequent increase in the rate of antibiotic formation, variable and inconclusive results were obtained. Supplementation of Base Medium A with a mixture of amino acids (containing lanthionine) gave no significant effects. Supplementation with a mixture of growth factors[4] gave varying results ranging from no apparent effect to moderate stimulation.

Since it had been demonstrated that the addition of an ash of asparagus butt juice to Base Medium C gave more rapid growth but variable antibiotic activities (2), the results were difficult to evaluate because effects obtained might have been caused by mineral impurities in the materials tested. However, slight to moderate increases in rate in addition to that given by the ash were given by supplementations with a variety of materials. For example, 0.4% yeast extract, which gave the most pronounced effect, almost doubled the antibiotic activities obtained at 48 hours.

Application of Results to Subtilin Production

Various phases of these studies have been applied to the production in submerged cultures on a laboratory (6) and a semi-pilot plant scale (8). Ammonia supplementation of a poorly processed asparagus press juice more than doubled the antibiotic activities. A partially synthetic medium gave rapid growth and relatively high activities.

The medium had the following composition: sucrose, 100 g.; citric acid, 11.7 g.; Na_2SO_4, 4.0 g.; yeast extract, 5.0 g.; $(NH_4)_2HPO_4$, 4.2 g.; Salts B (see Table II), 100 ml.; sufficient NH_4OH to adjust the pH to 6.8–6.9 (approximately 13 ml.), and water to give 1 liter. This medium was inoculated with 5% (by volume) of a blended surface culture grown on asparagus press juice (10% solids). The antibiotic activities obtained were 1500–2000 mg./l. in 10 hours in the laboratory fermenter operating with a 1 liter

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[4] The mixture of growth factors was added to give the following concentrations expressed in γ/ml. of the medium: Thiamine, 4; pyridoxine, 2; pyridoxamine, 2; calcium pantothenate, 4; riboflavin, 4; nicotinic acid, 2; nicotinamide, 2; p-aminobenzoic acid, 0.02; biotin, 0.02; folic acid, 0.02; adenine sulfate, 40; guanine hydrochloride dihydrate, 40; uracil, 40; xanthine, 40; inositol, 10; choline, 10.

charge of medium and 900–1200 mg./l. in 10–11 hours in a semi-pilot fermenter operating with 150 liters.

To demonstrate that the antibiotic activities of cultures grown on synthetic media were due to subtilin, the first two steps followed in the isolation (9) of subtilin were essentially employed on a culture grown on Base Medium C. A Fernbach flask containing 300 ml. of medium was inoculated and incubated for 4 days. The culture was blended, adjusted to pH 2.0 with dilute HCl, and blended again with 150 ml. of butanol. The butanol was separated by centrifugation and the activity was precipitated by diluting the butanol with 0.5 volume of diethyl ether. The antibiotic yields were: original culture, 120 mg.; butanol extract, 88 mg.; precipitate, 83 mg.

DISCUSSION

The difficulties attendant on nutritional studies of organisms with simple growth requirements have been discussed by Hutner (10). The physiology of these organisms should be considered as more complex than that of organisms with fastidious requirements, since the former must synthesize the materials that the latter require preformed, and it appears reasonable that the trace element nutrition of organisms with simpler requirements might influence the requirements for stimulatory organic materials. The ineffectiveness of lanthionine in promoting higher antibiotic activities was of interest, since lanthionine has been identified as a constituent of the subtilin molecule (11).

A comparison of the P, S, and N requirements with the mineral requirements studied (1, 2) (K, Mg, Fe, Mn, and Zn) is of interest

TABLE III

Comparative Requirements[a] for Essential Elements for Growth

	Approximate minimum requirement	
Element	p.p.m.	micro g.-ats./l.
N	1000	70,000
P	150	5,000
K	125	3,000
S	80	2,500
Mg	2.5	100
Fe	1.2	20
Mn	0.7	15
Zn	0.5	8

[a] These requirements were calculated from the results of a series of experiments on different media which gave different amounts of growth and are, therefore, linearly corrected to the basis of the production of 500 mg. of dried pellicle/50 ml. culture. The requirement for S was determined on Base Medium A; for P on Base Medium B; for N on Base Medium C plus an ash of asparagus press juice; and for K, Mg, Fe, Mn, and Zn on Base Medium A (2).

(Table III). In particular, K requirement was similar to P requirement and greater than S requirement. However, it should be emphasized that these requirements were calculated from results obtained on different media, at least one of which was experimentally demonstrated to be suboptimal.

The results of the studies on the P requirement (Fig. 2) were strikingly in contrast to the results of studies on the S and N requirements. Studies on the chemistry and physiology of the low and high P-containing cells, and on the remarkably constant percentage of P uptake by the cells, would be of great interest. The optimum concentration for maximum antibiotic activity found on Base Medium B was in close agreement with that reported in studies of the production of proteinase by *B. subtilis* var. *scaber* (12) in which a similar curve was obtained with an optimum range of PO_4 of 0.45–0.60 g./l. (150–200 p.p.m. of P). However, the fact that, in these studies, a similar P optimum for antibiotic activity was not found on other media indicated that this optimum range might have no relationship to the P physiology, and might be due to factors peculiar to the media under study, such as the relationships between the concentrations of the trace elements.

ACKNOWLEDGMENTS

SUMMARY

The nutritional requirements for the production of relatively high levels of subtilin by *Bacillus subtilis* in shallow-layer stationary cultures were found to be simple. In a medium properly balanced with respect to mineral salts, they were limited to an appropriate source of energy and to inorganic sources of N, P, and S. The quantitative requirements for the latter elements were determined.

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Studies on the Mineral Nutrition of the Subtilin-Producing Strain of *Bacillus subtilis*

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INTRODUCTION

In a study of the nutritional factors conducive to the production of the antibiotic subtilin by *Bacillus subtilis* (1), it was found necessary to initiate an extensive investigation of the mineral nutrition of the organism. This paper is limited to observations on requirements of the elements K, Mg, Mn, Zn, and Fe; the beneficial effects of citrate; the beneficial and deleterious effects of Ca and Cu salts; and effects of unidentified trace elements. A detailed report on the requirement for Zn has been presented recently (2). Previous investigations on the mineral requirements of various strains of *B. subtilis* have demonstrated or indicated that Mn, Fe, K, Ca, and Cu were either essential or stimulatory, and that organic acids, such as citric acid, were important constituents of the media employed (3, 4, 5, 6, 7, 8, 9).

MATERIALS AND METHODS

The culture used was the non-adherent stock previously employed (1, 2, 10). The general methods of preparation of media, culturing, antibiotic assays, etc., were as previously described (1). Base Media A and C used in this study were the same as those described in Ref. 1, Table II, with the exception that Base Medium A contained no citric acid. Purification of Base Medium A for the removal of Fe was achieved by extractions with chloroform solutions of 8-hydroxyquinoline (11). Cultures were incubated for 69–72 hours unless otherwise indicated. The minimum requirements for the various elements were considered to be the lowest concentrations at which the plotted curves leveled off.

RESULTS

Essential Elements

It was found necessary to include salts of K, Mg, Fe, Zn, and Mn in both Base Media A and C. Omitting any of these elements from Base

Medium A, either singly or as combinations, caused reductions in antibiotic activity of 80–95%, and in growth of 30–95%. Base Medium A was employed for the quantitative studies described below, since it gave the more consistent results and was apparently more deficient in the minerals under study. Also, Base Medium C contained a high concentration of citrate, which has been shown to influence markedly the quantitative mineral requirements of other bacteria (12).

Manganese

In Fig. 1 are presented the results of an experiment on the quantitative requirements for Mn. Similar requirements for Mn were observed in 3 other experiments, but considerable variations in the minimum
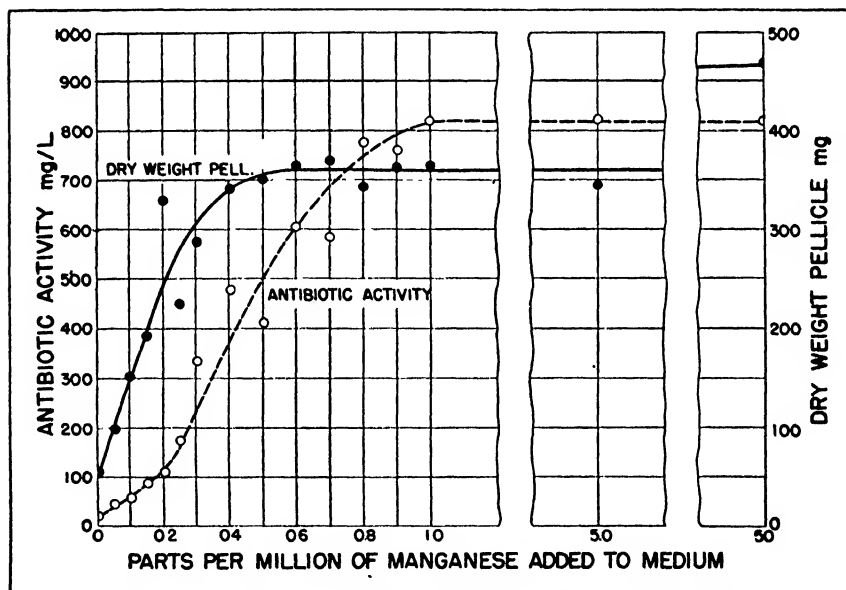
FIG. 1. The effect of Mn on growth and antibiotic activity. This experiment was performed on Base Medium A with Mn omitted from the salt mixture. Graded amounts of Mn added as $MnCl_2 \cdot 4H_2O$.

requirements for maximum antibiotic activity were found. None of 14 elements tested was capable of replacing Mn. The elements Cd, Ga, Al, Ni, Sn, Tl, Co, Mo, Cu, Ba, Pb, Hg, Sr, Cr, and B were tested as mixtures giving 1.0–1.5 p.p.m. of each element in the presence and

TABLE I

Spectrographic Analysis of Pellicle[a]

| Element | Amount element | | In pellicle |
	Added	Found	
	γ	γ	*Per cent*
Copper	None	70[b]	0.013
Manganese	250	70[b]	0.013
Iron	250	150[b]	0.029
Magnesium	500	150[c]	0.029

[a] Experiment was performed on Base Medium A with the Mg content reduced to 10 p.p.m. The dry weight of the pellicle obtained was 520 mg.

[b] Estimated accuracies of these analyses were ±20%.

[c] Estimated accuracy of this analysis was ±10%.
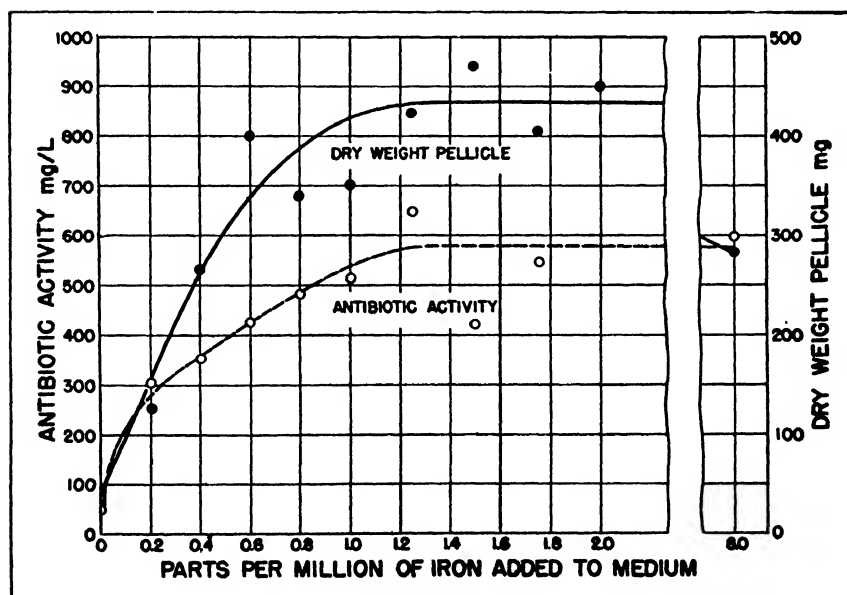
FIG. 2. The effect of Fe on growth and antibiotic activity. This experiment was performed on Base Medium A which had been extracted with chloroform solutions of 8-hydroxyquinoline according to Waring and Werkman (11) to remove the Fe. Graded amounts of Fe were added as $FeCl_3 \cdot 6H_2O$.

absence of adequate Mn. By visual estimation of the growth responses, this mixture would not replace Mn and did not influence the heavy growth obtainable in the presence of adequate Mn. The results of a spectrographic analysis of a pellicle are listed in Table I.

Iron

The results of a similar quantitative experiment with Fe, performed on a purified lot of medium, are presented in Fig. 2. In an experiment on the unpurified medium the minimum requirement was approximated at 1 p.p.m. by extrapolation.

None of 13 elements tested (Cd, Ga, Al, Ba, Sr, Cu, Ni, Sn, Co, Mo, Ca, Pb, and Tl) was capable of substituting for Fe. They were tested as mixtures giving 1.5 p.p.m. of each element, in the manner in which tests of substitution for Mn were performed.
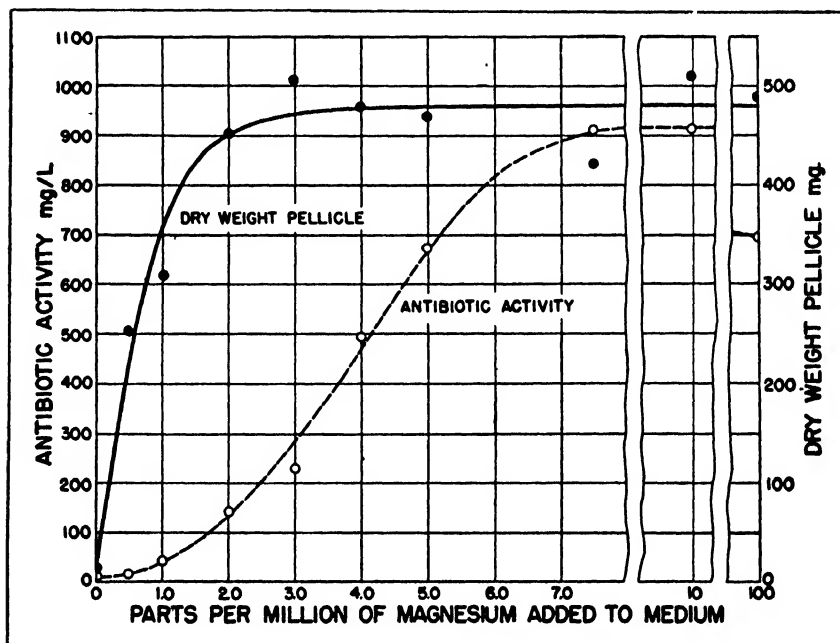
FIG. 3. The effect of Mg on growth and antibiotic activity. This experiment was performed on Base Medium A with Mg omitted from the salt mixture. Graded amounts of Mg added as $MgCl_2 \cdot 6H_2O$.

Magnesium

The greatest difference between the minimum requirements for growth (app. 2.0 p.p.m.) and for antibiotic activity (6.0–8.0 p.p.m.) was found with Mg (Fig. 3). The decrease in antibiotic activity with 100 p.p.m. of added Mg was consistently obtained.

Table II lists results of spectrographic analyses of pellicles from

TABLE II

The Magnesium Content of Pellicles[a]

Dry weight pellicle	Magnesium				
	Added		Found[b]	In pellicle	Recovery[c]
mg.	*p.p.m.*	*γ*	*γ*	*Per cent*	*Per cent*
260	0.5	25	37	0.014	150
460	2.0	100	100	0.021	100
480	4.0	200	150	0.031	75
470	5.0	250	110	0.023	44
430	7.5	373	150	0.034	40
520	10	500	150	0.029	30

[a] Experiment was performed on Base Medium A with Mg omitted from the salt mixture incorporated into the medium.

[b] The Mg determinations were made spectrophotometrically on ashes of the dried pellicles. The estimated accuracy of these analyses was ±10%.

[c] Calculated by dividing the amount of magnesium found in the pellicle by the amount added to the medium × 100.

cultures with graded amounts of added Mg. Despite the 3- to 4-fold higher Mg requirement for antibiotic activity than for growth, no correlation was noted (within the limits of the data) between the percentage of Mg in the pellicles and the antibiotic activities of the cultures. Ca and Be, the elements most closely related chemically to Mg, would not substitute for Mg when tested at concentrations of 1–10 p.p.m.

Potassium

Responses to graded additions of K are given in Fig. 4. Antibiotic activities in flasks with suboptimal levels of K were similar after 3 and 18 days of incubation (Table III).

None of the 3 elements most closely related chemically to K was found capable of replacing K. Na was a major constituent of the
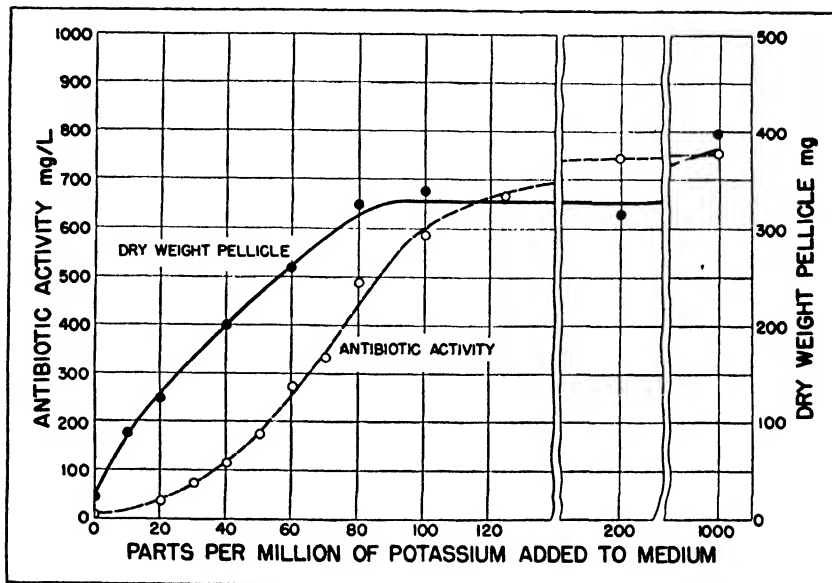
FIG. 4. The effect of K on growth and antibiotic activity. This experiment was performed on Base Medium A with K omitted from the salt mixture. Graded amounts of K added as KCl.

medium. Cs, tested at 100 and 400 p.p.m., did not support growth in the absence of K and did not affect the antibiotic activity obtained in the presence of adequate K. However, Rb replaced K almost quantitatively mole per mole for growth, but not for the production of antibiotic activity (Table III).

Zinc

Since the requirement for zinc on a purified medium had been previously reported (2), only its essentiality and approximate optimum concentration were reinvestigated. By visual estimation of the growth on Base Medium C, the growth was reduced approximately 50% in the absence of Zn and the requirement for growth was >0.25 p.p.m. and <0.50 p.p.m. Antibiotic activities on Base Medium A were reduced approximately 80% by omitting Zn from the salt mixture.

Citrate

In the presence of sufficient amounts of Ca and/or Mg to cause appreciable precipitation on Base Medium A, radical effects on the

TABLE III

Substitution of Rubidium for Potassium[a]

Expt. no.	Additions		72-hour harvest		18-day harvest
	Potassium	Rubidium	Dry weight pellicle	Antibiotic activity	Antibiotic activity
	$mM/l.$	$mM/l.$	mg.	mg./l.	mg./l.
	0.00	0.00	20	<20	
	0.51	0.00	120	<36	
	1.02	0.00	200	120	
	1.53	0.00	260	270	
	2.55	0.00	340	580	
1	5.10	0.00	320	750	
	25.5	0.00	400	740	
	0.00	0.58	110	<20	
	0.00	1.17	210	<20	
	0.00	4.67	420	72	
	25.5	1.17		620	
	25.5	4.67		620	
	0.00	0.00		~20	30
	1.02	0.00		230	200
	2.55	0.00		470	470
	5.10	0.00		750	610
2	0.00	1.17		<20	<30
	0.00	4.67		89	<60
	1.02	1.17		340	220
	1.02	4.67		380	220
	5.10	4.67		650	510

[a] Experiment was performed on Base Medium A with K omitted from the salt mixture incorporated into the medium.

antibiotic activities were obtained. In general, levels which caused such precipitations caused reductions in antibiotic activities but not necessarily reductions in growth, although marked differences in the physical appearances of pellicles were usually noted. Even the level of Mg routinely used in the medium (50 p.p.m.) sometimes gave slight reductions in antibiotic activity. Ca gave results similar to, or more marked than, those obtained with Mg, and the deleterious effects were additive when the Ca concentration was the same as, or greater than,

the Mg concentration. Results of an experiment are presented in Table IV. In contrast to these additive effects were results in which deleterious effects by Ca were partially prevented by the addition of very high (250–500 p.p.m.) levels of Mg.

The addition of citrate prevented the precipitations caused by Ca and Mg and also prevented the associated reductions in antibiotic activities. With low levels of Mg or Ca, citrate was unnecessary to obtain good growth and relatively high antibiotic activity, but usually

TABLE IV

Interrelationships Between Magnesium, Calcium and Citrate[a]

Additions		Antibiotic activities	
Magnesium	Calcium	No citrate	0.2% citrate
p.p.m.	*p.p.m.*	*mγ./l.*	*mγ./l.*
0	0	20	20
10	0	910	1200
100	0	700	1200
0	10	20·	20
10	10	1000	1300
100	10	800	1200
0	100	20	20
10	100	620	1300
100	100	320	1200

[a] Base Medium A was employed with the Mg omitted from the salt mixture incorporated into the medium.

gave a small beneficial effect on antibiotic activity (Table IV). Whether or not citrate was utilized was not determined.

Anomalous reductions in antibiotic activities were obtained by increasing the Mn concentration 10-fold (to give 50 p.p.m.) in the presence of citrate and a high level of Ca on Base Medium A (Table V).

The quantitative requirements for the essential elements on Base Medium A with added citrate or on Base Medium C were not determined. However, doubling the salt mixture in Base Medium C did not materially affect growth or antibiotic activity.

Unidentified Essential or Stimulatory Elements

Conclusive evidence was obtained that the mineral composition of Base medium C was inadequate. Definite but variable increases in the

TABLE V

Interrelationships Between Manganese, Calcium and Citrate[a]

Additions of calcium and citrate	Antibiotic activities			
	Experiment A		Experiment B	
	p.p.m. Mn	p.p.m. Mn	p.p.m. Mn	p.p.m. Mn
	5.0	50.0	5.0	50.0
	mg./l.	*mg./l.*	*mg./l.*	*mg./l.*
1. None	850	860	660	690
2. Ca. 100 p.p.m.	– –	—	230	240
3. Citrate, 0.2%	980	1100	1200	1000
4. No. 2 + No. 3	940	270	800	310

[a] Experiment was performed on Base Medium A with additional Mg to give a total of 100 p.p.m.

rate and extent of growth were found with the addition of ashes of asparagus press juice or yeast extract. Although limited efforts to reproduce the effects given by the ash on Base Medium C were unsuccessful, some responses were obtained with Ca and Cu. Heavier and more rapid growth was given by the addition of 10–50 p.p.m. Ca in 5 separate experiments, but the degree of stimulation varied considerably and very erratic results were obtained in an experiment on quantitative requirements. In one experiment the dry weights of the pellicles were 610 mg. without added Ca and 880 mg. with 25 p.p.m. added Ca. Similar but less extensive effects on antibiotic activities were obtained. Cu, tested at 0.5–1.5 p.p.m., gave pellicles which appeared firmer and heavier but which had weights similar to the controls without added Cu. Antibiotic activities with added Cu were generally lower than in its absence. These beneficial effects by Ca and Cu were not found on Base Medium A.

DISCUSSION

In this and a recent investigation (2) it was considered that the elements Fe, Mn, Zn, Mg, and K were demonstrated to be essential for the growth of the subtilin-producing strain of *B. subtilis* on the media studied. The specificity of requirements for Fe, Mn, and Mg, the replaceability of Zn only by Cd and only to a limited extent, and the

replaceability of K only by Rb and only for growth, are in marked
contrast to the nonspecific effects noted by others (13, 14). Data
obtained with Ca and Cu were not considered sufficiently satisfactory
to warrant the conclusion that these elements are either essential or
stimulatory. Hutner (13) aptly pointed out the difficulties in evaluating
results with Ca salts. But the beneficial effects given by the ashes and
by salts of Ca on Base Medium C, although variable, were sufficiently
great to demonstrate that the medium was not optimal.

The data did not support the hypothesis that citric or similar
organic acids are necessary for the utilization of inorganic nitrogen (9).
The deleterious effects of large amounts of Ca and Mg on Base Medium
A were attributed to the precipitations caused by these elements.
These precipitations were interpreted as rendering essential elements
less available, either by chemical precipitation or by physical occlusion
on the Ca and Mg precipitates, and their prevention by the addition
of citrate was attributed to the formation of soluble complexes. The
peculiar beneficial effects given by large amounts of Mg on the deleteri-
ous effects of Ca in the absence of citrate might be attributed to changes
in the physical or chemical nature of the Ca precipitate so that less of
the essential elements was precipitated. Obviously, other interpreta-
tions for these phenomena are feasible.

Calculated on a similar basis (the minimum concentration necessary
to give 500 mg. of dried pellicle/50 ml. of culture) the requirements of
the various elements were approximately (in p.p.m.): K, 125; Mg, 2.5;
Fe, 1.2; Mn, 0.7; Zn, 0.5. These requirements are much higher than
those found for many bacteria. For example, the Fe requirements of
Aerogenes indologenes and *Pseudomonas aeruginosa* were reported (11)
as 0.03 and 0.09 p.p.m., respectively, and the Mn requirements of
several lactic acid bacteria were reported as approximately 0.1 p.p.m.
(calculated) (12). Variations in the quantitative requirements between
different experiments were probably due, at least in part, to unavoid-
able variations in the rates of growth. This phenomenon was previously
reported with Zn, where it was demonstrated that the Zn requirement
for the production of antibiotic activity was a function of the time of
incubation (2). No reason was found for the large difference between
the Mn requirements on the beet molasses media (10) and Base Media
A and C. This difference in requirement may be due to factors similar
to those discussed by McLeod and Snell (12); in their work the quanti-

tative requirements of lactic acid bacteria were influenced by the concentration of citrate.

In general, the results obtained with K were distinctly different from those obtained with the other four elements and appeared to support the hypothesis that a main function of K in the organism's physiology is noncatalytic in nature. The molar replacement of Rb for K for growth but not for antibiotic production may be of value in determining the mode of synthesis of antibiotic by the organism. The K and Rb salts tested might have differed, in that the K salt might have contained a mineral impurity necessary for subtilin formation. More probably, although Rb is capable of almost complete substitution for K, it is incapable of substituting for at least one function for which K is necessary.

This strain of *B. subtilis* should prove especially appropriate for further studies of the mineral nutrition of a bacterium, particularly under submerged culture in which very rapid growth is possible (15).

ACKNOWLEDGMENTS

SUMMARY

The elements K, Mg, Fe, Mn, and Zn were demonstrated to be essential for growth and antibiotic formation by *Bacillus subtilis*. The requirements of these elements for the production of 500 mg. of dried pellicle per 50 ml. of culture were approximately (in p.p.m.): K, 125; Mg, 2.5; Fe, 1.2; Mn, 0.7; and Zn, 0.5. The requirements for the formation of antibiotic activity were generally higher than the requirements for growth. The element Rb was found to replace K for growth but not for antibiotic formation.

Beneficial effects were obtained by the addition of citrate, particularly to media in which precipitations occurred.

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Lipoxidase Activity and Fat Composition of Germinating Soy Beans

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INTRODUCTION

In connection with this laboratory's investigations on the nature of soy bean lipoxidase and its action (1, 2), it was of interest to gather some information on the function of this enzyme in the plant's metabolism. Although the enzyme's function is presumably related to the oxidation of the linoleic and linolenic acids present in relatively large quantities in the oil of the soy bean, little information is as yet available to indicate when this action takes place in the life cycle of the plant, or to what changes in fat composition its action is correlated. It was thought that perhaps lipoxidase action may have some relation to the well-known loss of fat in germinating seeds, so it was decided to study first the changes in the composition of germinating soy beans to discover the relationship between lipoxidase activity and such changes as occur.

EXPERIMENTAL

Agat soy beans, obtained from Algot Holmbergs Co., Norrköping, which tested 99% germination, were planted in May in soil in a shallow box and placed in a sunny place. The plants were watered daily, and, as the experiment proceeded, portions of the plot were removed as samples for analysis. The plants were washed free of soil and immediately frozen. The frozen plants were chilled further by submersion in liquid air, and, while they were at this low temperature and extremely brittle, they were ground finely in an ordinary coffee mill. The ground samples were then dehydrated while in the frozen condition. The dried samples were then weighed and extracted for 2 days with pentane in Soxhlet extractors. Pentane was chosen as solvent because hydrocarbons do not destroy lipoxidase activity, whereas ether does, and because a low-boiling solvent was desired to reduce the possibility of heat-

inactivation of the enzymes. The weighed fat samples were stored under carbon dioxide at $-15°C$. until used.

The absorption spectra of the fat samples were determined in pentane solution using the Beckman spectrophotometer. The iodine values were determined using the Hausman method and duplicate determinations were made whenever samples were large enough to permit.

The linoleic and linolenic acid contents of the fat samples were determined by the alkaline conjugation method (3). Fat samples of approximately 100 mg. were weighed into small glass vials (5 × 5 mm.) and dropped into tubes containing 5.0 ml. of 11% KOH in ethylene glycol which had been preheated to 180°C. for 15 minutes. After shaking vigorously twice during the first few minutes of the reaction, the samples were left at 180°C. for a total of 30 minutes, at the end of which the tubes were chilled quickly by plunging them into ice water. The cooled samples were diluted appropriately with methanol, and the light absorption at 2680 and 2340 Å was measured using the Beckman spectrophotometer. Blanks and standards of linoleic and linolenic acids were run simultaneously.

The fat-extracted dried soy bean meal was pulverized and 100 mg. was suspended in 10.0 ml. $M/15$ secondary sodium phosphate solution with a Potter homogenizer. The homogenate was centrifuged and the clear portion used for lipoxidase assay using the spectrophotometric assay (4), and for determination of catalase activity (5).

RESULTS AND DISCUSSION

Representative individuals of each of the 11 samples taken for analysis during the experiment are shown in Fig. 1. A portion of sample 11 was separated into tips, leaves, stems, cotyledons, and roots for separate analyses. The means of separation is also indicated in Fig. 1. The data obtained from the various analyses are shown in graphic form in Fig. 2 to facilitate comparisons. In Figs. 2A and 2B the fat, linoleic acid, and linolenic acid contents of the soy bean samples are expressed as per cent of the dry weight. In Fig. 2C the lipoxidase content is expressed as units/mg. of dry, fat-extracted meal; and the catalase content is expressed as K/mg. of dry, fat-extracted meal, where $K = \log_{10} \left(\frac{H_2O_{2_0}}{H_2O_{2_t}} \cdot \frac{1}{t} \right)$.

The fat content of the dehydrated soy bean meal samples remained constant for the first two days, showed a slight increase the third day, and then showed a steady decline for the duration of the experiment. A similar slight increase in fat content the first two days of germination was noted by Neumann (6). The subsequent decrease in fat content of various germinating seeds has been described by several workers (6–11), and in studies of *ricinus* seeds Houget (12) has attrib-
fat loss to synthesis of sugars. Johnson and Sell (13) came to

conclusions regarding the germination of tung kernels. Inasmuch as the total dry matter of germinating soy beans decreases, the decrease in fat content of the dried beans represents a total loss of fat.

The iodine values of the fat of the germinating seeds remained constant until after the fifth day, when a decrease in the unsaturation of

Fig. 1. Soybean samples 0–11 taken during an 18-day growing period. The method of division of sample 11 is indicated.

the total lipids was noticed. Unfortunately, the total yield of fat also decreased, prohibiting duplicate determinations after this time, so that the values in the latter part of the experiment cannot be taken as more than an indication of the trend. In studies on germinating soy 1 white beans, Neumann (6) found the same slow decrease e first six days, but Shuiku (8) reported an increased iodine
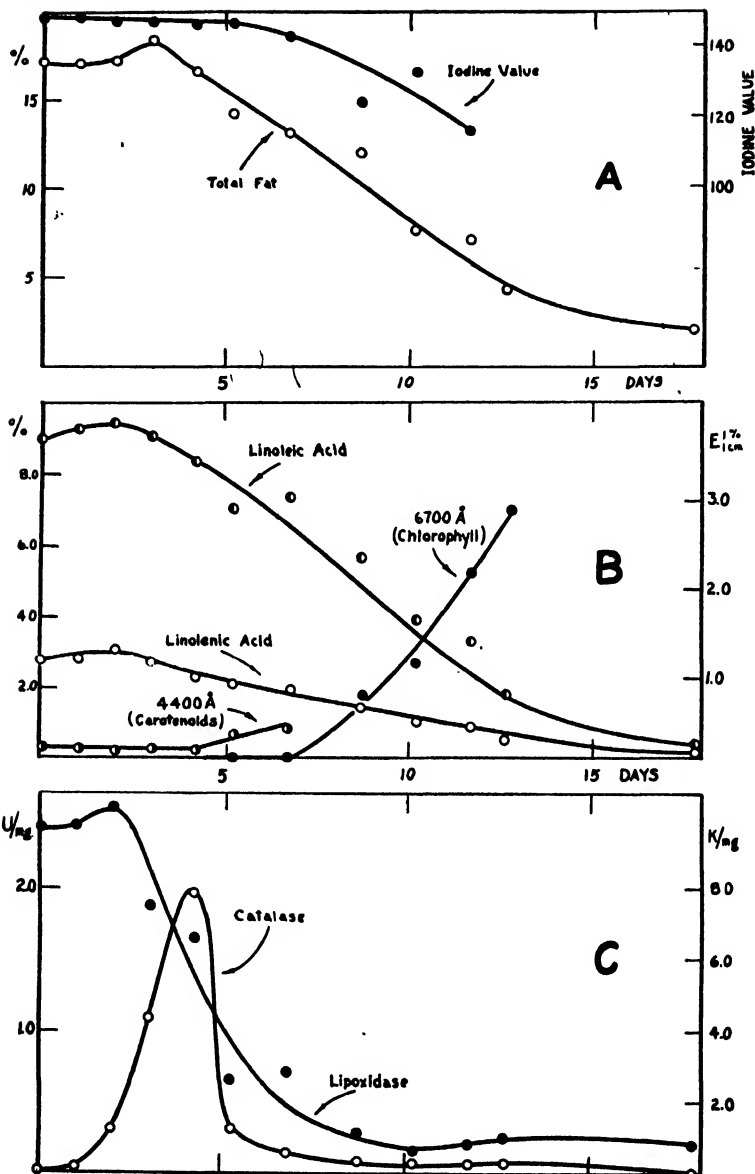
FIG. 2. Changes occurring during germination of soy beans. A. Iodine value; fat expressed as per cent of dry matter. B. Linoleic and linolenic acids expressed as per cent of dry matter; light absorption of oils at 4400Å (carotenoids) and 6700 Å (chlorophyll) expressed as $E_{1cm}^{1\%}$. C. Lipoxidase expressed as units/mg. fat-free dry matter; catalase expressed as K/mg. fat-free dry matter.

value of the oil of germinating soy beans. McLachlan (7) found that the cotyledon fat of germinating soy beans remained of the same degree of unsaturation, but that the fat synthesized in the growing plant tissue was more saturated than the original oil.

The linoleic acid and linolenic acid contents of the dry bean plants showed a definite decrease after the third day. This decrease is not merely a reflection of the decrease in total fat, for the content of these acids in the oils also showed a significant decrease. Thus, linoleic acid decreased from approximately 52% to 23% of the oil during the 18-day germination period, and a parallel decrease in linolenic acid from 16% to 12% was observed. It thus appears that, although the total fat decreases sharply, there is a preferential decrease of the linoleic and linolenic acids. These results are not in agreement with those of Shuiku (8), who reported an increase in thiocyanogen value during germination, presumably indicating an increase in these acids.

From the absorption spectra of the fat samples some information can be obtained regarding the carotenoid and extractable chlorophyll contents of the beans. The carotenoid contents of the oils, as measured by the absorption of light at 4400 Å, remained constant through the fourth day, but showed significant increases beginning on the fifth day. Similarly, the extractable chlorophyll content, as measured by the absorption of light at 6700 Å, remained constant near zero through the seventh day, after which time the beans began emerging from the soil. It is of interest to point out that the chlorophyll appeared after the increase in carotenoids, indicating that the increased absorption at 4400 Å represented a true appearance of pigment other than chlorophyll.

It was hoped that the measurement of light absorption at 2300 and 2700 Å in the oils would give some clue as to the activity of lipoxidase, for the oxidation products of linoleic and linolenic acids are known to show strong absorption in these regions (14, 15). The light absorption of the oils remained low at these wavelengths until the fourth day, when the first increases were observed. Beyond that time the presence of pigments in the oils complicates the interpretation of the data. From the data available at present it is impossible to state whether conjugated oxidation products of linoleic and linolenic acids accumulate to any degree in the oils of germinating soy beans.

The catalase activity of seeds has been known to show a sharp maximum early in the period of germination (16, 17). The association

of high catalase concentration and fat in both plants (18) and animals (19) hints that catalase may function in some phase of fat metabolism. From the sharp maximum in catalase activity around the fourth day after planting, it would appear that this function, if real, is of very short duration, and occurs at the phase where lipoxidase activity is in sharp decline.

Results of analysis of the various parts of sample 11 are shown in Table I. An examination of the data reveals that lipoxidase activity is

TABLE I
Composition of Parts of the Soy Bean Plant

	Total plant	Tip	Stem	Leaf	Coty-ledon	Root
Per cent fat (of dry matter)	2.0	3.0	1.3	4.12	3.7	0.70
Per cent linoleic acid (of fat)	22.6	18.6	11.5	—	31.3	6.4
Per cent linolenic acid (of fat)	11.6	10.8	9.4	—	13.6	5.9
$E_{1cm.}^{1\%}$ 4400 Å	18.6	13.2	10.5	—	22.7	2.1
$E_{1cm.}^{1\%}$ 6700 Å	2.2	4.2	4.6	—	9.5	0.3
Catalase K/mg.	0	0.4	0	0	0	0
Lipoxidase U/g.	8.1	65.0	79.0	66.0	392.	56.0

highest in the cotyledon where fat, linoleic acid, linolenic acid, and fat-soluble pigments are also highest. Catalase was detected only in the tips at this stage of the plants' growth.

The results gained in this investigation do not explain the function of lipoxidase in the metabolism of the soy bean, but they do prompt some speculations. The high lipoxidase activity in the dormant bean and the sharp decline in lipoxidase activity during the early stages of germination suggest that lipoxidase is either stored in the bean in an inactive form or is isolated from its substrate, which also occurs in the bean in large amounts. The absorption of water by the bean may allow contact between the enzyme and its substrate, initiating the oxidation of the linoleic and linolenic acids.

Van Fleet (20) has shown that, in a variety of crop plants, oxidase is high at the seedling stage in fatty tissues, but this oxidase activity was low at the end of 5 weeks, at which time the fat content was low. It was expected that liposidase would increase in activity at the time when it began to function, as indicated by the disappearance of its substrate. However, it is apparent that the enzyme activity suddenly decreases

after initiating the oxidation of linoleic and linolenic acids. This may indicate that the enzyme is inactivated as a consequence of its action. This has been indicated by *in vitro* experiments of several workers, under a variety of experimental conditions. This products-inactivation of the enzyme has been found to be markedly increased with increasing temperature. Contrary to the general findings of Van Fleet, lipoxidase activity was very low at the seedling stage in soy beans. It may well be that the Van Fleet test for oxidase activity detects fatty acid oxidases other than lipoxidase.

However, prolonged high activity of lipoxidase should not be necessary for prolonged oxidation of its substrates, for it should be remembered that the methylene-interrupted polyunsaturated fatty acids oxidize autocatalytically. Once the reaction is initiated, the oxidation can proceed by a chain mechanism. It may thus be that lipoxidase has its function only in initiating the oxidation of the unsaturated fatty acids during the germination of the soy bean.

Acknowledgments

Summary

The changes in fat content, iodine number of fat, linoleic and linolenic acid contents of the fat, chlorophyll and carotenoids, lipoxidase and catalase have been followed during the germination of soy beans.

Carotenoids begin to increase on the fifth day, chlorophyll on the seventh. Fat content shows a decrease after the third day, iodine value of the oil decreases after the fifth day. Linoleic acid and linolenic acid contents decrease after the second day, and the loss of these acids is proportionately greater than the loss of total fat.

Catalase activity shows a very sharp maximum at the fourth day. Lipoxidase activity declines sharply after the second day, at the time its substrates being to disappear. It may be that the function of lipoxidase is to initiate the oxidation of linoleic and linolenic acids which then can proceed autocatalytically.

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Interrelation between α-Tocopherol and Protein Metabolism. III. The Protective Effect of Vitamin E and Certain Nitrogenous Compounds against CCl₄ Poisoning in Rats [1]

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INTRODUCTION

In a previous paper (1) we reported that vitamin E increased the utilization of casein for growth in young rats. The effect was seen on diets containing 10% casein but not at casein levels over 14%. In any case, the beneficial effect of α-tocopherol on growth and protein efficiency was small although significant statistically. A criterion was needed which would be more dramatic and reliable than growth differences. Possibly any physiological response which shows up more intensely at lower levels of dietary casein would be critically influenced by the presence of vitamin E.

The susceptibility of rats to carbon tetrachloride poisoning is known to be greater at low dietary casein levels. Forbes and Taliaferro (2) have shown that a diet of carrots protected against exposure to CCl₄, and an earlier paper from this laboratory (3) showed that α-tocopherol could replace a carrot diet in protecting rats against anoxic anoxia. These facts suggested that a study of the influence of α-tocopherol on carbon tetrachloride toxicity, might throw further light on the relation between vitamin E and protein in the nutrition of the rat.

EXPERIMENTAL

Groups of young male albino rats were fed one of the vitamin E-free diets listed in Table I. Supplements, to be described later, were given to various groups for periods ranging from 3 weeks to 10 weeks.

TABLE I

Composition of Diets

Ingredient	583	P 583	58
Vitamin Test Casein[a]	--	10	—
Crude Casein	10	—	22
Sucrose	76	76	64
Salt Mixture, USP No. 2[b]	4	4	4
Lard	10	10	10
Vitamin Supplement[c]			

[a] General Biochemicals, Inc.

[b] With added $ZnCO_3$, 0.1%; $CuSO_4 \cdot 7H_2O$, 0.08%; and $MnSO_4$, 0.01%.

[c] Added to casein to give,/g. diet: thiamine, riboflavin, pyridoxin, 10 γ each; calcium pantothenate, 25 γ; choline chloride, 1 mg.; i-inositol, 0.1 mg.; vitamin A, 50 units; vitamin D (Delsterol), 3 units. Folic acid (Lederle's Folvite) added to some diets at 10 γ/g.

The rats were then injected intraperitoneally with pure carbon tetrachloride at a level of 2 cc./kg. body weight. Preliminary tests had established that this level of CCl_4 would kill 80% or more of the unsupplemented control rats on diet P-583 (10% casein). Carbon tetrachloride at 1.5 cc./kg. killed between 10 and 60%, while 1.0 cc./kg. killed less than 20% of the rats. Most of the deaths following a single injection occurred within 2 days. A study of liver pathology was not considered worthwhile in these rats because of their rapid deaths. Less acute intoxications, using liver damage as the criterion, will be the subject of future experiments.

Supplements of d-α-tocopherol were fed, usually at a level of 1 mg. daily, in a solution of olive oil. Other supplements were added to the diet, usually at 0.1% level.

RESULTS

Low-Casein Diets

The data in Table II show the protective effect of d-α-tocopherol against the lethal action of carbon tetrachloride. Of the unsupplemented E-free rats only 13 out of 49 rats (26%) survived 2 days, while with vitamin E supplementation 30 out of 34 rats (88%) survived.

Neale and Winter (4) have shown that xanthine protects rats against liver damage and death following carbon tetrachloride exposure. We have studied the protective action of xanthine and related compounds when added as supplements to the vitamin E-free diet, P-583.

The data given in Table II show that xanthine had a moderate but definite protective action under these conditions. Yeast nucleic acid was better; guanine had activity about equal to xanthine, as did

TABLE II

The Protective Effect of α-Tocopherol and Certain Purines and Amino Acids against
CCl₄ Poisoning in Rats on a 10% Casein Diet

Exp.	Diet	Supplement	Days on supple-ment	Average body weight	Average gain	Survivals 48 hr. after CCl₄ at 2 cc./kg., i.p.	
				g.	*g.*		
I	P-583		70	137	87	1/9	(11%)
	P-583	1 mg. α-tocopherol daily	70	148	102	9/9	(100%)
II	P-583		42	116	68	8/25	(32%)
	583		42	149	100	2/5	(40%)
	P-583	1 mg. α-tocopherol daily	42	115	65	12/15	(80%)
	583	1 mg. α-tocopherol daily	42	138	90	5/5	(100%)
	P-583	4% yeast, dry	42	128	77	2/5	(40%)
	P-583	3% liver, dry	42	123	80	3/5	(60%)
	P-583	0.1% xanthine	42	112	66	3/5	(60%)
	P-583	0.1% nucleic acid	42	118	69	4/5	(80%)
III	P-583		25	89	30	2/10	(20%)
	P-583	1 mg. α-tocopherol daily	25	94	35	4/5	(80%)
	P-583	0.1% xanthine	25	99	33	2/5	(40%)
	P-583	0.1% hypoxanthine	25	100	31	0/5	(0%)
	P-583	0.1% guanine	25	101	37	2/5	(40%)
	P-583	0.1% histidine	25	95	29	2/5	(40%)
	P-583	0.1% glycine	25	89	30	1/5	(20%)
	P-583	0.1% arginine	25	101	37	0/5	(0%)

histidine. However, hypoxanthine was inactive, as were glycine and arginine.

The influence of dimethylxanthines and of d-γ-tocopherol on the lethal action of carbon tetrachloride are presented in Fig. 1. The moderate action of xanthine is greatly enhanced by methylation at the 1,3-position as in theophylline, but is lessened by methylation at the 3,7-positions as in theobromine. d-γ-Tocopherol has only a fraction of the activity of d-α-tocopherol.

The influence of 6-methylthiouracil was interesting. As can be seen in Fig. 1, thiouracil negated the beneficial action of 1 mg. α-tocopherol daily. Whether higher tocopherol levels would be effective awaits future experiments. By itself thiouracil was non-protective.

Table III summarizes the relative protective activities of various compounds when fed as supplements to the vitamin E-free 10% casein diet.
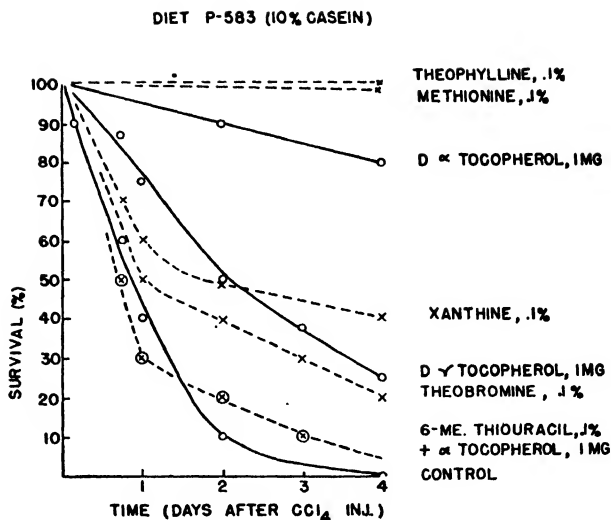
DIET P-583 (10% CASEIN)

FIG. 1. Influence of supplements to diet P-583 (10% casein) on survival after carbon tetrachloride poisoning. Each group contained 8–10 rats averaging 90–100 g. CCl_4 was injected intraperitoneally at a level of 0.2 cc./100 g. body weight.

The data of Fig. 2 show that d-α-tocopherol succinate mixed in the diet at 0.02% was as effective as d-α-tocopherol fed by dropper. These data also show that tocopherol dimer[2] was inactive at 5 mg. daily. The dimer lessened the protective action of α-tocopherol at the levels fed. Whether higher levels of tocopherol will overcome this depressant action has not yet been tested.

An experiment designed to determine the influence of the level of α-tocopherol is reported in Fig. 3. The duration of the lethal power of

TABLE III

Classification of Compounds with Respect to Protection of Rats,
on a 10% Casein Diet, against CCl_4 Toxicity

Highly protective	Moderately protective	Non-protective
d-α-Tocopherol	d-γ-Tocopherol	Tocopherol dimer
DL-Methionine	Xanthine	Hypoxanthine
Theophylline	Guanine	Theobromine
	Histidine	Thiouracil
	Nucleic Acid	Glycine
		Arginine

[2] Obtained from Dr. J. G. Baxter. This compound formed from two molecules of γ-tocopherol, has one tocopherol equivalence by the Emmerie-Engel test.

the single dose of carbon tetrachloride is surprisingly long. At the lowest level of tocopherol supplement, discontinued at the time of poisoning, good protection was afforded for 2 days, but after this "lag phase" deaths ensued rapidly. Continued tocopherol supplementation prevented the delayed deaths. The data in Fig. 3 indicate that storage levels of α-tocopherol laid down in the prepoisoning period greatly minimizes the lethal response to the single dose of carbon tetrachloride.
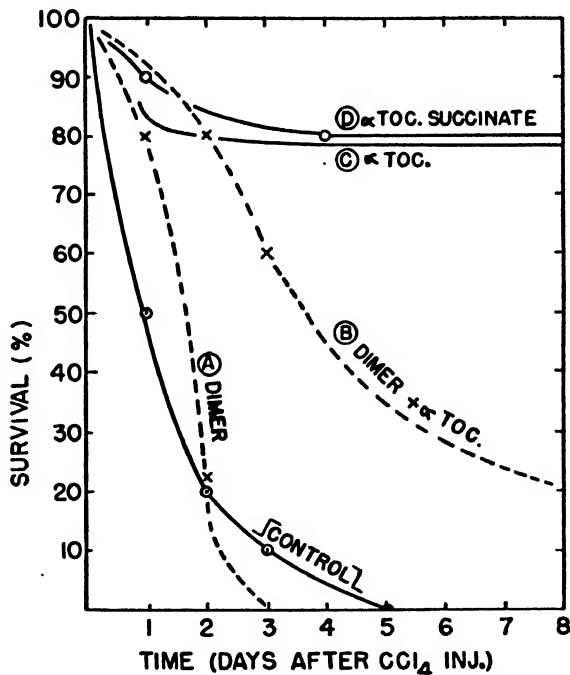
Fig. 2. Influence of α-tocopherol and tocopherol dimer on survival of rats after carbon tetrachloride poisoning. Diet P-583 (10% casein) was used. Curve (A): 5 mg. of tocopherol dimer daily. Curve (B): Same, plus 1 mg. d-α-tocopherol. Curve (C): 1 mg. d-α-tocopherol daily. Curve (D): d-α-tocopherol succinate added to diet at 0.02%.

Normal Casein Level Diet

Rats fed the vitamin E-free diet containing 22% casein were relatively resistant to carbon tetrachloride at the level used. Nor did methionine or d-α-tocopherol afford additional protection. On the other hand, xanthine did confer added protection, while benzimidazole
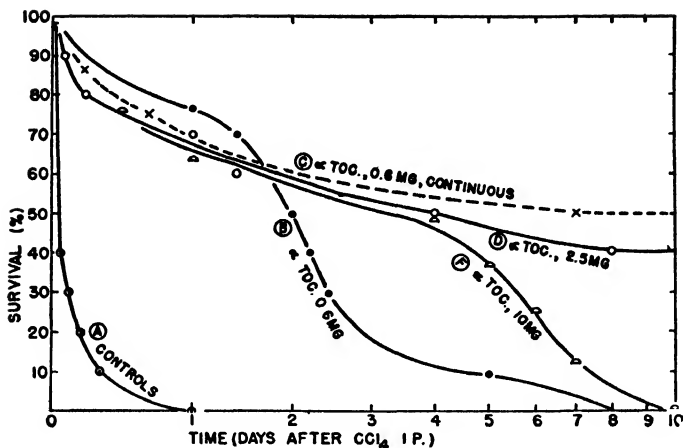
FIG. 3. Influence of amount and continuity of d-α-tocopherol supplements on survival of rats after carbon tetrachloride poisoning. Each group contained 8–10 males on Diet P-583 (10% casein). The indicated α-tocopherol supplements were given daily up to the time of poisoning. The animals making up Curve (C) were continued on supplement after poisoning.
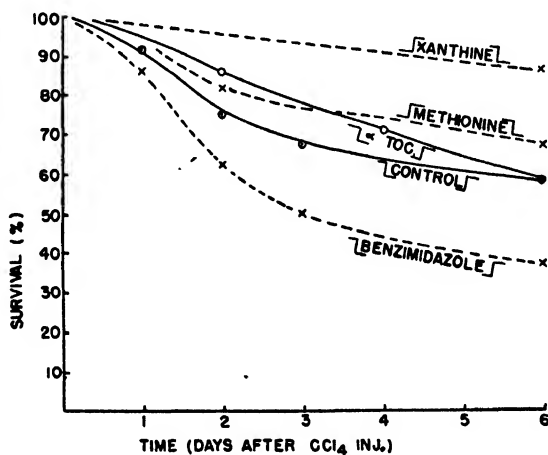
FIG. 4. Influence of supplements to normal (22%) casein diet on survival of rats after carbon tetrachloride poisoning. d-α-Tocopherol was fed at 1 mg. daily. The other supplements were included in the diet at 0.1%. Each group contained between 8 and 12 male rats. Carbon tetrachloride was injected intraperitoneally at a level of 0.2 cc. /100 g. body weight.

acted in the opposite direction by increasing the death rate. These results are shown in Fig. 4.

DISCUSSION

The action of d-α-tocopherol in protecting rats against carbon tetrachloride toxicity is more dramatic and clear-cut than the vitamin E influence on growth (1). In both cases the effect was seen when a 10% casein diet was used but was not evident in rats on diets with higher casein levels.

Aside from the theoretical interest in the relation between organic solvent toxicity and vitamin E, this relationship offers a technique which is rapid and has a clearly defined endpoint. Its possible use as a bioassay procedure for the E-vitamins may be worth investigating. d-γ-Tocopherol was considerably less active than d-α-tocopherol; this is the same relative activity which has been found for most other manifestations of the vitamin E deficiency state, such as resorptive sterility (5), muscular dystrophy (6), or pigment formation (7).

Of peculiar significance is the fact that methionine or theophylline can replace α-tocopherol in protecting rats against carbon tetrachloride. Until more is known about the mechanism of carbon tetrachloride poisoning, little can be said about mechanisms for protection.

However, it is known that all 3 compounds are active antioxidants, *in vitro*. This property of α-tocopherol is well known. Recently, it has been shown that small amounts of methionine will more than triple the antioxidant activity of tocopherol (8). Giri and Rao (9) have shown that purine compounds with a replaceable hydrogen atom at the 7-position in the imidazole nucleus are good antioxidants for vitamin C protection. This may explain the high activity of theophylline as contrasted with its isomer theobromine. All of the methylated xanthines are said to possess strong lipotropic activity (10).

SUMMARY

A study of protective agents against carbon tetrachloride toxicity in rats on a 10% casein, vitamin E-free diet showed that:

1. d-α-Tocopherol gave excellent protection whether fed by dropper as 1 mg. daily supplement, or included in the diet as the succinic acid ester. d-γ-Tocopherol fed at the same level was much less active than the α-form,

2. Theophylline (1,3-dimethylxanthine) gave excellent protection, replacing α-tocopherol in this action. Xanthine and guanine were moderately active. Hypoxanthine and theobromine (3,7-dimethylxanthine) were inactive.

3. DL-Methionine also gave good protection. Glycine and arginine were inactive. Increasing the casein level in the diet (to 22%) gave good protection, on top of which supplements of methionine or α-tocopherol gave no added benefit.

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Fat Synthesis in Yeast

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INTRODUCTION

Smedley-MacLean and coworkers (1, 2, 3) have established the conversion of acetic acid into fat and sterols by yeast. Recently, it was shown, by the use of C^{13}, that the long chain fatty acids of *Saccharomyces cerevisiae* can be formed by utilization of intact added acetate without prior conversion to carbohydrates (4). The purpose of this communication is to extend this work and to discuss the experimental conditions under which maximum and reproducible increases in fat can be obtained by non-proliferating yeast cells with acetate as the sole source of carbon.

METHODS

The non-proliferating cell suspension technic was used throughout. *Saccharomyces cerevisiae*, carried on glucose agar slants and transferred once a month to maintain viability, was used in these experiments. The culture was kept at 4°C. This organism has been shown (3) to have a strong anaerobic metabolism and appears to be a typical strain of the species.

Large batches of cells were prepared for metabolic experiments by transferring an inoculum from the slant with a sterile loop to 200 ml. of sterile medium (glucose, 1%; K_2HPO_4, 0.1%; NH_4Cl, 0.1%; Difco malt extract broth, 1.5%). Incubation was at 30°C. for 24 hours. The 200 ml. were then poured aseptically into 10 liters of the medium in a 12-liter flask. After growth for 24 hours the cells were harvested by centrifugation in a Sharples centrifuge at 80,000 r.p.m. Aliquots of yeast paste were weighed and transferred either to 300 ml. or 1 l. cylindrical glass test-tubes.

Aeration was by means of 1 inch alundum balls attached to glass tubing and inserted to the base of the tube. Air, freed of CO_2 by bubbling through 20% NaOH, served as a source of oxygen. The gas was passed through the medium at the rate of

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0.5 volume/min., as determined by the rate of displacement of a known volume of water.

The suspending medium used throughout was 0.025 M or 0.25 M phosphate buffer, pH 7.0, prepared according to Clark (5). Sodium acetate, when added, was the commerical C.P. grade. Since the pH of the acetate in aqueous solution is about 8.5, it was brought to 7.0 with 10% HCl before addition to the medium. The pH measurements were made either with 0.04% bromthymol blue indicator or on a Coleman pH meter.

The procedure for preparation of the cell suspensions for analysis after the nonproliferating incubation period was the same in all cases. The cells were removed from the suspending medium in a Swedish "angle" centrifuge and washed twice with water. Two to 20 ml. of water were used, depending upon the initial weight of cells. The washings were added to the supernate which was used for residual acetate and other determinations. The cells were degraded as outlined below.

The total fat content of the yeast was determined according to Smedley-MacLean (6). The cell paste was suspended in N HCl equivalent to 5 times the wet weight of the cells. The cells were hydrolyzed by boiling gently under reflux for 2 hours. The hydrolyzed cell suspension was then neutralized to phenolphthalein with NaOH and filtered hot. The precipitate was washed twice with hot water and dried in a desiccator under vacuum. After drying, the precipitate was transferred to a Soxhlet extractor and extracted with ether for 24 hours. The ether solution was transferred with washing to a dry, weighed, 100 ml. round bottom flask, the ether removed *in vacuo* and the quantity of fat determined by drying and reweighing the flask. A 24 hour extraction is adequate for recovery of the hydrolyzed fat; a further 24 hour extraction results in an increase of less than 1% of the total fat.

EXPERIMENTAL

The effect of cell age on fat synthesis was determined in a series of experiments in which cells of 18, 24, 48, 72, and 96 hrs. suspended in 0.025 M phosphate at pH 7.0 were used. Phosphate served to buffer as well as to provide for the formation of energy-rich phosphate bonds (7, 8, 9). The cells were harvested by centrifugation, suspended in 300 ml. of buffer containing 30 mM of sodium acetate and aerated for 48 hrs. at 30°C. when they were removed, washed, and hydrolyzed. Total lipide was determined.

The results are given in Fig. 1. It has been shown by Smedley-Mac-Lean and Hoffert (1, 2) that the ratio of sterols to fat remains constant during the increase in total lipide in the presence of acetate. We have confirmed these observations. An increase in total lipide may, therefore, be considered as representative of a corresponding increase in the fat fraction. Two controls were run with each series of experiments: one 12 g. sample of cells was allowed to metabolize in the absence of acetate and a second 12 g. aliquot was analyzed for total lipide at zero time. The fat of the first aliquot served as the reference value from which the

increase in fat could be calculated. Unless otherwise noted, all values given are the average of at least 3 determinations. The error among determinations was not in excess of 10%.

Optimum fat production occurs with 24 hour cells. An adequate explanation for these results may be obtained when we consider the formation of fat to involve the storage function of the cell. During the early stages of growth the building of new protoplasm rather than fat
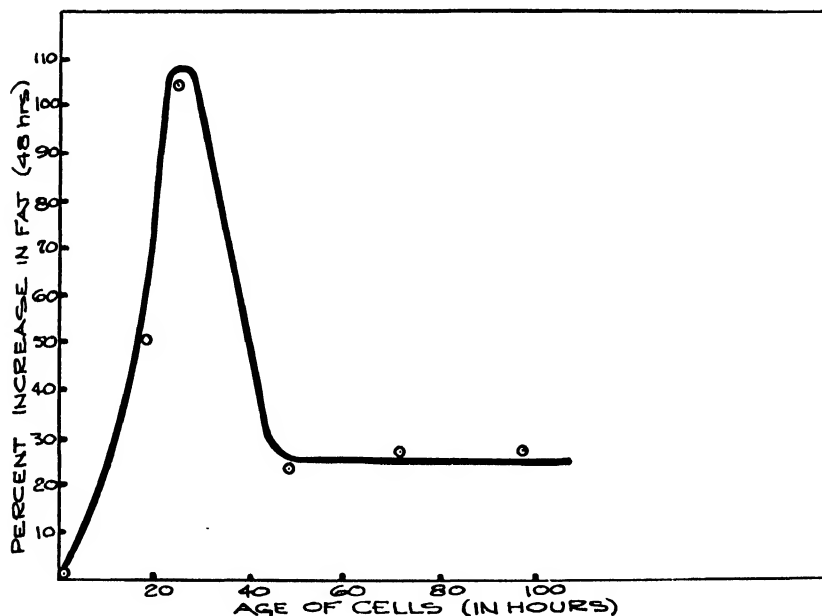
Fig. 1. Effect of age of cells on fat synthesis.

is the primary process. When maximum, or near maximum, growth has been obtained, the storage function will assume greater importance. Although our data do not offer proof of this suggestion, it has been noted that no marked increase in the total weight of cells is obtained after 24 hours. The decline in the synthesis of fat between 24 and 48 hours can be most satisfactorily explained as a decrease in synthetic activity coupled with an increase in the proportion of dead to living cells.

We were unable to find, as did Smedley-MacLean and Hoffert (1, 2), consistent increases in fat of between 50 and 100% in cells

suspended in aerated phosphate buffer alone. In general, the increases obtained under our conditions without acetate were less than 10%.

Effect of Time of Aeration on Fat Synthesis

Since a 48 hr. aeration had been found satisfactory in the experiments of Smedley-MacLean and Hoffert (1, 2), the same time was employed for the first series of experiments. The optimum aeration period was determined in a series of experiments in which 12 g. of 24 hour cells were supsended in 0.025 M buffer with and without added acetate. Fifty ml. aliquots were withdrawn from each flask at 12, 24, 36, 48, 60, and 72 hours after aeration was begun. The cells were removed, hydrolyzed, and dried. Equal weights of the dried cellular material were taken for ether extraction.

An aeration time of 24–48 hours gave maximum fat synthesis (Fig. 2). The rapid decline in stored fat after 48 hours may be correlated with

FIG. 2. Effect of time of aeration on fat synthesis.

disappearance of the added acetate as will be noted in the next series of experiments. Since the source of material for fat synthesis had thereby been removed, the cells could conceivably utilize the stored fat as an energy source and remove it by oxidation more rapidly than it could be synthesized from the cellular carbohydrate.

Effect of Acetate on Fat Synthesis

There are no reports in the literature on the effect of a concentration of acetate less than 0.1 M, although Smedley-MacLean and Hoffert (1, 2) reported a lowering of the amount of fat synthesized with concentrations above 0.1 M. The effect of acetate concentrations less than 0.1 M was studied. Since it had been noted in earlier experiments that the pH of the medium rose markedly because of the liberation of Na^+, the concentration of phosphate buffer was increased to 0.25 M.

Four g. wet weight of cells in 100 ml. of the suspending medium were used throughout. Sufficient sodium acetate was added to each tube to
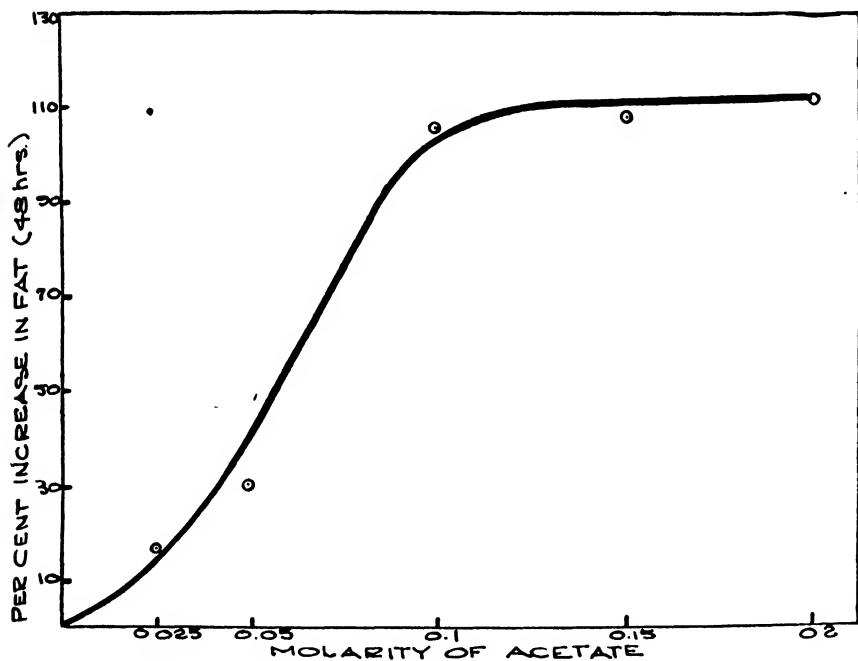
FIG. 3. Effect of concentration of acetate on fat synthesis.

give a final concentration of 0.025, 0.05, 0.15, and 0.2 M. There seems to be a slight increase in the amount of fat formed with 0.2 M acetate as compared with 0.1 M (Fig. 3) and not a decrease as had been reported. A correlation appears to exist between the concentration of acetate added below 0.1 M and the amount of fat produced; 0.025 M gave almost one-half the increase of fat given by 0.05 M and one-fourth

the value of 0.1 M These results are to be expected if the acetate molecule is directly synthesized into fat as we have previously shown (5). The results obtained with the higher concentrations of acetate may be variously explained. The liberated sodium ion may have a detrimental effect on the enzymes concerned in fat synthesis as shown for lower concentrations of Ca^{++} and Mg^{++} by MacLeod and Smedley-MacLean (3). Secondly, the 0.1 M acetate solution may saturate the enzyme systems and, under prolonged aeration, a larger increase in stored fat might have occurred with the higher concentrations.

Effect of pH on Fat Synthesis

Four g. samples of wet cells were used per tube. Phosphate buffers, 0.5 M, or pH 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0, were prepared and checked on a Coleman pH meter. An aqueous solution of sodium acetate was adjusted to a pH comparable to the buffer in which it was to be used with 1 N HCl. The final concentration of the acetate was 0.2 M. Fifty ml. each of acetate solution and of buffer were then mixed to give a phosphate concentration of 0.25 M and an acetate concentration of 0.1 M. The pH was then rechecked, the cells were added and aeration was begun.

At an initial pH between 6.5 to 8.0, there appears to be no marked difference in the amount of fat formed (Table I). Below 6.5 the amount

TABLE I

Effect of pH on Fat Synthesis by Yeast

pH		Without acetate	With 0.1 M acetate	Increase
Initial	Final			
		mg. fat	*mg. fat*	*Per cent*
7.0	7.0	21.0		
5.5	5.7		22.7	8.1
6.0	6.3		29.6	41.0
6.5	6.8		44.1	110
7.0	7.6		45.2	115
7.5	8.2	.	44.7	113
8.0	8.9		45.7	118

Each flask contained 4 g. wet wt. of cells, 100 ml. of 0.25 M PO$_4$ buffer at pH given. Aeration time, 48 hours.

of fat synthesized falls off sharply. No values were obtained above pH 8.0. Since a final pH of 8.0 was usually obtained with the 0.2 M acetate in the previous experiments, doubt is cast upon the deleterious effect of the liberated sodium ions.

It is of interest to note that, in normal yeast fermentation, an increase in pH above neutrality leads to an increase in the production of acetate while a decrease brings about increased formation of ethyl alcohol (10).

Synthesis of Fat by Dried Yeast Preparations

Many metabolic experiments can be carried out successfully by the use of either dried or lyophilized preparations as indicated by the work of Krampitz and Werkman (11, 12), among others. The use of this procedure when possible simplifies the non-proliferating cell experiments for one can prepare large enough batches of cells for many experiments. The following experiments were conducted with both dried and lyophilized 24 and 48 hour suspensions of the organism.

The dried preparations were made by spreading the wet cell paste, obtained after incubation in the growth medium, on porous clay plates and drying in a vacuum desiccator; lyophilized cells were prepared by use of a lyophilizing apparatus based on the original design of Flosdorf and Mudd (13). The procedure consisted of freezing a thin layer of wet organisms on the inside surface of a round bottom flask, attaching the flask to a high vacuum system which contained an acetone-dry ice trap to remove the vaporized water. When dry, the cells were powdered very easily and stored dry until use.

Five g. of each type of preparation were used for these experiments. The yeasts were first suspended in 300 ml. of 0.25 M phosphate buffer and shaken until well dispersed. Sodium acetate was then added to the test suspensions and the suspension aerated for 48 hours. Fat analyses were made after the incubation period (Table II).

TABLE II

Fat Synthesis by Dried Yeast Preparations

Types of preparation	Without acetate	With 0.1 M acetate	Increase
Lyophilized cells	*mg. fat* 260.5	*mg. fat* 271.1	*Per cent* 3.3
Air-dried cells	244.0	212.0	−13.1

Each flask contained 5 g. of dried cells, 300 ml. of 0.025 M PO₄ buffer, pH 7.0. Aeration time, 48 hours.

Dried preparations were obviously not satisfactory for our purpose; the specific metabolic disturbance has not been determined. On transfer of the lyophilized organisms to a glucose agar slant, growth occurred.

Conclusions

The conditions under which a relatively large and reproducible increase in fat may be obtained using non-proliferating cells of *S. cerevisiae* have been described: (1) A 24 hr. culture of cells. (2) An aeration time of between 24 and 48 hours. (3) An acetate concentration of 0.1 *M*. (4) An initial pH of between 6.5 and 8.0.

Dried and lyophilized cells do not show the synthetic ability of freshly harvested cells.

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Pantothenic Acid Studies. III. A Pantothenic Acid Conjugate Active for *Acetobacter suboxydans* [1]

In the course of a series of investigations dealing with pantothenic acid conjugates from animal tissues and yeasts, we have found a material in heart muscle which appears to possess activity for *A. suboxydans* greater than that of free pantothenic acid in a pantothenic acid-deficient medium (1). The activity of the new material is far higher than can be accounted for by the pantothenic acid present, as measured by *L. arabinosus* after digestion with takadiastase and papain (2). That the substance contains pantothenic acid is strongly suggested by the fact that no compound is known to be able to substitute for the vitamin (or pantoic acid) in media in which it is lacking, and also by the observation that the β-alanine content, as determined by yeast (3), increases upon acid hydrolysis. These observations are recorded in the accompanying table.

The activity for *A. suboxydans*, although stable to enzyme digestion, is easily destroyed by acid; sample 210 below on standing with 6 N HCl for one hour at 40°C. lost virtually all of its growth-promoting power. After 6 hours hydrolysis, the β-alanine values roughly account for half of the activity of the conjugate in *A. suboxydans*. This suggests that the conjugate may be about twice as active as the free vitamin for this organism.

The demonstration of greater activity by a conjugate than by the free vitamin has been observed in *Hemophilus influenzae* or *parainfluenzae* with coenzyme I or II, where nicotinic acid or amide has virtually no growth-promoting power (4). The existence of bound forms of pantothenic acid has also been reported, and experiments are

TABLE I

The Apparent Pantothenic Acid Activity of Heart Muscle Concentrates
for L. arabinosus, LM Yeast and A. suboxydans[a]

Sample no.	A. suboxydans		LM yeast			L. arabinosus	
	Treatment						
	None	Enzyme[b] digested	None	Enzyme digested	Acid[c,d] hydrolyzed	None	Enzyme digested
	γ/mg.						
210	2.12		0.44		1.10	0.085	0.05
300	1.93					0.026	0.037
502	2.90					0.057	0.058
600	1.35	1.1	0.11	0.13	0.81	0.053	0.099
601	1.62	1.84	0.073	0.082	0.57	0.076	0.080

[a] In terms of calcium pantothenate.
[b] 2% Papain and takadiastase (2).
[c] Refluxed with 10 volumes of 6 N HCl for 6 hours, then removal of HCl.
[d] β-Alanine standard used; pantothenate value calculated.

in progress to determine the possible identity of any of these with the present growth factor. The conjugates reported by Neal and Strong (5) and Wright (6) are probably different, for the former is reportedly alkali-stable, while the latter is decomposed by heat and is precipitated by protein reagents. The properties of the present factor seem similar to those of coenzyme A (7) as reported by Lipmann and coworkers and Guirard, although attempts to acetylate sulfanilamide using the present conjugate have thus far been unsuccessful.

Concentrates of the pantothenic acid conjugate possess glutamic acid activity (8), in the ratio of about one mole of pantothenic acid (calculated from the β-alanine value) to 2–4 moles of glutamic acid. Studies dealing with isolation of the conjugate and determination of its structure are in progress.

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The Activation Mechanism and Physicochemical Properties of *Clostridium histolyticum* Proteinase

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INTRODUCTION

Proliferating cells of *Clostridium histolyticum* produce and secrete into the culture medium a proteinase, the activity of which is enhanced by the presence of heavy metal and sulfhydryl compounds (1, 2). Increased activity in the presence of these compounds appears to be typical for proteinases of the strict anaerobes (3).

This study[2] yielded a simple method of purification of the *Cl. histolyticum* proteinase, by means of which the enzyme was obtained as an apparently homogeneous protein with practically the same activation behavior as the purified enzyme. The experiments here reported provide further indication that the full, as well as the initial, activity of this proteinase is to be ascribed to the action, not of two different enzymes (4, 5) but of one and the same protein (2, 3).

This purified enzyme, with gelatin and clupein as substrates, was used to explore the mechanism of activation. The observation was made that the unactivated enzyme has a clearly limited range of hydrolysis which can be considerably increased by simple addition of heavy metal and sulfhydryl compounds.

The purified enzyme gave no positive test for sulfhydryl or disulfide groups, and was shown spectrographically to contain very little tyrosine or tryptophan.

EXPERIMENTAL

As in previous work (1), a dissociated strain of *Cl. histolyticum* No. 4972 of the American Type Culture Collection was used for preparation of the enzyme. A 3% neopeptone (Difco) solution of pH 7.4 was inoculated with culture freshly grown in

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[2] Most of the experimental work was done during the fall of 1938.

the same medium, then incubated anaerobically at 37°C. for 16–18 hours. At this point, incubation was terminated to prevent autolysis of the bacteria (2). The culture was immediately filtered through a Berkefeld filter, and the proteinase isolated as described under *Purification*. The proteolytic activity was measured by determining the increase in free amino groups produced in a standard protein substrate (gelatin Gold Label) by the van Slyke method (1, 2). By initial activity is meant the original proteinase activity without added activator, by full activity the activity obtained in the presence of an $Fe^{++}-SH$ activator ($FeSO_4$-cysteine) (1, 2). The enzyme preparations varied in activity from time to time, apparently because of variations in the growth and consequent proteinase secretion of the bacteria.

Purification

Since contamination by peptidases, which are liberated by autolysis of the bacterial cell, had been prevented by restricting the period of incubation, the purification of the proteinase could be followed, assuming a zero order reaction, by the relation:

$$\frac{\text{Activity in ml. } 0.1\ N\ \text{KOH}}{\text{Dry weight} \times \text{hours of incubation at 37°C.}}.$$

To 2 liters of the dark brown bacteria-free enzyme solution sufficient anhydrous potassium sulfate (6) was added (at 38°C.) to precipitate all the protein. On dialysis through cellophane against running water for 24 hours, the precipitate dissolved to a clear brown liquid about 200 ml. in volume. In the Tiselius apparatus (7) the pigment and other impurities in this crude concentrate were found to migrate more rapidly than the enzyme. Electrophoresis, therefore, provided a simple means for the isolation of pigment-free enzyme, apparently homogeneous with respect to migration. Because of the limited capacity of the apparatus, however, enzyme purified in this manner was reserved for experiments in which absence of impurities was most essential.

TABLE I

Control of Purification by Measurements of Full Activity and Dry Weight

Determination: 2 ml. (or 1 ml.) enzyme solution, 5 ml. citrate-phosphate buffer pH 7.0, 3 ml. 6.6% gelatin solution adjusted to pH 7.0, 2 ml. $Fe^{++}-SH$ activator (= 0.6 mg. $FeSO_4$ and 10 mg. cysteine-HCl) adjusted to pH 7.0, and 1 ml. (or 2 ml.) water; incubated at 37°C.

	Volume analyzed	Dry weight	Incubation	Activity in ml. of 0.1 N KOH	Activity / (Dry weight × hours of incubation at 37°C.)
	ml.	*mg.*	*hours*		
Crude bacterial filtrate	2	45	20	4.02	0.00045
K_2SO_4-precipitated enzyme	1	20	1	1.70	0.085
Acetone-precipitated enzyme	1	11	1	1.90	0.17
Electrophoretically separated enzyme	1	5.7	1	2.70	0.47

For other experiments, the crude concentrate was purified by acetone precipitation. The addition to the chilled solution of an equal volume of cold acteone caused precipitation of the enzyme after standing for a short time in the refrigerator. The precipitated enzyme dissolved to a clear yellow solution of unchanged activation behavior. The purification effected by these precedures is shown in Table I.

ELECTROPHORETIC PURIFICATION AND PHYSICOCHEMICAL PROPERTIES

Correlation of Electrophoretic Pattern with Proteolytic Activity

The crude enzyme concentrate was examined in the Tiselius apparatus (7) with the simple schlieren optical system, in citrate-phosphate buffer solutions of ionic strengths 0.1 and 0.02 at pH 5 and pH 8, and of ionic strengths 0.5, 0.1, and 0.02 at pII 7. The pattern consisted regularly of a sharp boundary, B, of low mobility, preceded by a broad boundary, A, which was clear-cut only in the most concentrated solutions, and which coincided with the pigment boundary. Table II shows

TABLE II

Proteolytic Activity of Electrophoretic Fractions of K_2SO_4-precipitated Cl. histolyticum Proteinase. Comparison with Acetone-purified Proteinase

Determination: 1 ml. enzyme or fraction, 5 ml. citrate-phosphate buffer pH 7, 3 ml. 6.6% gelatin solution adjusted to pH 7, and 2 ml. water; incubated one hour at 37°C.

Fraction	Components present	Proteolytic activity (ml. of 0.1 N KOH)			
		Initial	+Fe^{++}	+SH	+Fe^{++}−SH
1. Unfractionated solution	A, B, Pigment	1.26	1.92	1.76	2.72
2. Ascending arm (upper section)	A, Pigment	0.01	0.00	0.01	0.01
3. Descending arm (upper section)	B	1.26	1.93	1.80	2.70
Acetone-precipitated enzyme		0.74	0.88	1.00	1.72

that proteolytic activity was associated with B (fraction 3, descending arm). The activity and dry weight of this, the electrophoretically separated enzyme, indicated a purification of about 1000 times with respect to the crude filtrate (Table I).

The pigment and A, devoid of activity themselves, apparently inhibited the activity of the enzyme, for, although the crude concentrate (fraction 1 of Table II) and the purified enzyme (fraction 3) were practically identical in activity, the enzyme concentration of the latter,

determined from the position of the boundary in the electrophoresis cell at the close of the experiment, was only 75% as great as in the former. The data in Table II show that in spite of this inhibition the activation behavior was unaffected by the impurities, and was the same for the crude concentrate as for the electrophoretically separated enzyme. The behavior of the acetone-purified product was similar.

Electrophoretic Properties

At each pH the mobility of the proteinase was found to increase, rather than decrease, with increasing ionic strength. This anomalous behavior, which is also characteristic of pepsin, is probably to be explained by the combination of the proteinase with anions of the medium (8).

In later experiments, using the Longsworth method (9), the mobility was consistently -0.9×10^{-5} cm./sec. volt/cm. in phosphate-saline buffer solution of pH 6.7, ionic strength 0.1, and -1.3×10^{-5} in citrate-phosphate buffer solution of pH 7.4, ionic strength 0.1. Dia-
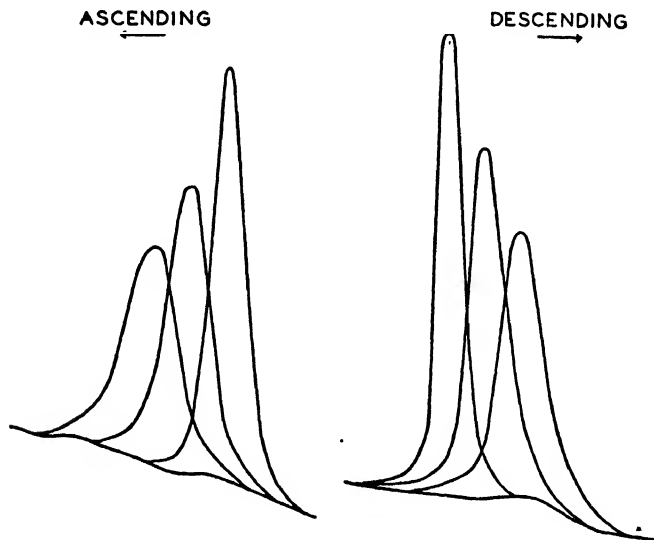
Fig. 1. Electrophoresis diagrams for electrophoretically purified *Clostridium histolyticum* proteinase (B) in phosphate saline buffer solution of pH 6.7, ionic strength 0.1. Concentration (dry weight): 10.5 mg./ml. Migration for 4, 6, and 8 hours at a potential gradient of 5.1 v./cm.

grams for the electrophoretically purified enzyme in the former medium are shown in Fig. 1. The preparation was apparently homogeneous, even after prolonged migration. There was similar evidence for homogeneity from pH 5 to pH 8. Measurements of curve area and dry weight indicated a specific refractive increment of about 0.0015.

Absorption Spectrum

The absorption spectra of the electrophoretic fractions of the potassium sulfate-precipitated proteinase were determined with Hilger echelon cells (10) and a quartz spectrograph. The curves are reproduced in Fig. 2: (1) refers to the unfractionated solution, (2) to a mixture of A and pigment, free from enzyme, and (3) to the enzyme faintly tinted with pigment. A completely colorless, more dilute solution of enzyme
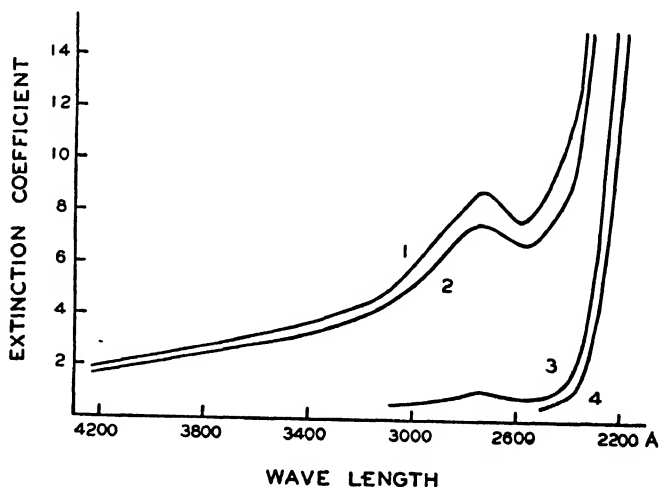
FIG. 2. Ultraviolet absorption spectrum of *Clostridium histolyticum* proteinase.

Curve	Electro-phoresis	Fraction	$\Delta n \times 10^4$ (refractometer)	Constituents present
1		Unfractionated, K₂SO₄-precipitated	13	Enzyme (B), A, pigment
2	pH 7, ionic strength 0.1	Ascending arm, lower section	3.5	A, pigment
3		Descending arm, upper section	9	Enzyme (B), trace pigment
4	pH 8, ionic strength 0.1	Descending arm, upper section	7.5	Enzyme (B), free from pigment

gave curve 4. The high absorption from 2500 Å to 2900 Å which is characteristic of most proteins, and for which the residues of the the aromatic amino acids are responsible (11), was not shown by the pigment-free enzyme; the extinction coefficient was less than 0.3 at wave lengths longer than 2550 Å. Consequently, tyrosine, if present at all, constitutes less than 0.6% of the enzyme molecule, and tryptophan less than 0.25%.

INFLUENCE OF VARIOUS REAGENTS ON THE ACTIVITY OF *Clostridium histolyticum* PROTEINASE

In an effort to determine the groups with which the activity of *Cl. histolyticum* proteinase is associated, the activity of the acetone-purified enzyme was measured in the presence of various reagents. The results however (Table III) were not conclusive. The inhibition of the enzyme

TABLE III

Influence of Various Reagents on the Activity of Purified Clostridium histolyticum Proteinase

Determination as in Table II; 10 mg. of each reagent added. Activities expressed as ml. of 0.1 N KOH.

Initial activity of proteinase: 0.84			
Reagent	Activity	Reagent	Activity
Iodoacetic acid	0.68	p-Iodoacetanilide	0.84
Iodoacetamide	0.59	Sodium fluoride	0.56
Potassium iodate	0.85	Phenyl hydrazine	0.44
Sodium iodide	0.69	Semicarbazide	0.40
Iodine	0.45	Hydrazine	0.42
Iodoaniline	0.82	Maleic acid	0.85

by most of the iodine compounds might be interpreted to indicate dependence of activity upon the presence of sulfhydryl groups in the molecule. On the other hand, the failure of maleic acid to inhibit the activity might be considered proof for the absence of active sulfhydryl groups (12). The more decisive test with sodium nitroprusside (13), using 5 ml. of electrophoretically purified enzyme, gave results which were negative for both sulfhydryl and disulfide groups. The uniform and pronounced inhibition by aldehyde reagents seems to indicate the

participation of aldehyde or keto groups in the activity of the enzyme molecule (14).

DECOMPOSITION OF GELATIN BY *Clostridium histolyticum* PROTEINASE

The increased activity manifested by *Cl. histolyticum* proteinase upon the addition of heavy metal salts and sulfhydryl compounds raises the question of the mechanism by which the increase occurs. In a previous investigation (15) it was found that the adapted proteinases, gelatinase (which hydrolyzed only gelatin) and caseinase (which hydrolyzed only casein), were enabled, respectively, to hydrolyze casein and gelatin if ferrous salts and cysteine were added. These observations suggested that the initial enzyme has only a limited range of specificity toward the protein molecule, while the addition of Fe^{++} −SH broadens that specificity and extends the range of hydrolysis. The purified enzyme seemed well suited to a quantatative test of this hypothesis.

Gelatin was used first as substrate. Table IV summarizes the experimental details and the results. Gelatin was incubated with an excess of the initial enzyme; samples were withdrawn immediately after addition of the enzyme, and at intervals during the incubation, to determine the amino and carboxyl groups liberated (Table IV, A). When hydrolysis had ceased, a portion was treated with an equal volume of the same (initial) enzyme; after incubation for 3 hours, the resulting mixture showed no further liberation of amino and carboxyl groups.

The end-point for the reaction of the initial, unactivated enzyme having thus been established, the Fe^{++}−SH activator was added to a portion of the hydrolyzed gelatin. Samples for analysis were removed immediately following addition of the activator, and at intervals thereafter (Table IV, B). The end-point was reached in 3 hours; no further hydrolysis was induced by the addition of more of the enzyme, freshly activated.

A separate experiment was then carried out, with the enzyme and the activator added simultaneously to the gelatin (Table IV, C). The amino and carboxyl groups liberated by this, the fully activated enzyme, were, within the limits of error, the sums of those liberated by the initial enzyme before and after addition of the activator. The unactivated enzyme and the activated enzyme participated in the

TABLE IV

Hydrolysis of Gelatin by Clostridium histolyticum Proteinase

Reaction Mixture A: 70 ml. acetone-purified enzyme (initial proteolytic activity = 0.89, full activity = 1.52 ml. 0.1 N KOH; no peptidase or polypeptidase activity), 30 ml. 6.6% gelatin solution adjusted to pH 7.

Reaction Mixture B: 20 ml. of A, completely hydrolyzed, and 2 ml. of $Fe^{++}-SH$ activator (27.4 mg. $FeSO_4$ and 30 mg. cystine-HCl) adjusted to pH 7.

Reaction Mixture C: 70 ml. enzyme (same as for A), 30 ml. 6.6% gelatin solution, 2 ml. $Fe^{++}-SH$ activator (27.4 mg. $FeSO_4$, 30 mg. cysteine-HCl) adjusted to pH 7.

Incubation	Increase of free amino and carboxyl groups in 200 mg. gelatin (as ml. 0.1 N KOH) [a]					
	A Before addition of activator: initial enzyme		B Activator added when hydrolysis by initial enzyme was complete		C Enzyme and activator added simultaneously: fully activated enzyme	
	Amino[b]	Carboxyl[c]	Amino[b]	Carboxyl[c]	Amino[b]	Carboxyl[c]
hours						
1	0.47	0.64	0.86	0.98	1.35	1.95
4.5	0.65	1.28	1.21	1.29	1.79	2.64
6	0.65	1.28	1.22	1.30	1.79	2.68
Additional enzyme added	0.65	1.28	1.22	1.30	1.79	2.68

[a] These figures include the KOH added to compensate for the continuous drop in pH which begins immediately after addition of the substrate, and maintain a constant pH of 7.

[b] Determination in van Slyke micro apparatus, using 2 ml. of reaction mixture.

[c] Titration with alcoholic KOH (16), using 5 ml. of reaction mixture.

hydrolysis of gelatin in the ratio of about 1:1. The difference in titer between the amino and carboxyl groups indicates the liberation of imino groups, a finding consistent with the known high proline and hydroxyproline content of gelatin (17).

DECOMPOSITION OF CLUPEIN BY *Clostridium histolyticum* PROTEINASE

Since the break-down products formed by the hydrolysis of gelatin might exert on the enzyme an inhibitory influence, the extent of which could not be controlled, repetition of the above experiment with a more suitable substrate seemed desirable. The protamine clupein was selected for this purpose because it is readily hydrolyzed by the

proteinase, has a very low molecular weight, and can be prepared in relatively pure condition. As with gelatin, the figures for hydrolysis by the fully activated enzyme were the sums (Table V) of those for hydrolysis by the unactivated enzyme before and after addition of the activator.

TABLE V

Hydrolysis of Clupein by Clostridium histolyticum Proteinase

Reaction Mixture A: 10 ml. clupein sulfate (= 1472 mg.) adjusted to pH 7, and 40 ml. acetone-purified enzyme (initial proteolytic activity = 0.92 ml., full activity = 1.51 ml. 0.1 N KOH/hour at 40°C. on 200 mg. gelatin at pH 7; no peptidase or polypeptidase activity).

Reaction Mixture B: 20 ml. of A, completely hydrolyzed, and 2 ml. Fe^{++}—SH activator (= 27.4 mg. $FeSO_4$ and 30 mg. cysteine-HCl) adjusted to pH 7.

Reaction Mixture C: 5 ml. clupein sulfate (= 736 mg.) adjusted to pH 7, 20 ml. acetone-purified enzyme (same as for A), and 2 ml. Fe^{++}—SH activator (= 27.4 mg. $FeSO_4$ and 30 mg. cysteine-HCl) adjusted to pH 7.

Incubation	Increase of free amino and carboxyl groups in 200 mg. clupein (as ml. 0.1 N KOH) [a]					
	A Before addition of activator: initial enzyme		B Activator added when hydrolysis by initial enzyme was complete		C Enzyme and activator added simultaneously: fully activated enzyme	
	Amino[b]	Carboxyl[c]	Amino[b]	Carboxyl[c]	Amino[b]	Carboxyl[c]
hours						
1	0.74	1.31	0.78	0.72	1.49	2.40
4	0.91	1.88	0.98	0.95	1.95	2.92
6	0.92	1.89	0.98	0.95	1.95	2.89
Additional enzyme added	0.92	1.89	0.98	0.95	1.95	2.92

[a] These figures include the KOH added to compensate for the continuous drop in pH which begins immediately after addition of the substrate, and maintain a constant pH of 7.

[b] Determination in the van Slyke micro apparatus, using 2 ml. of reaction mixture.

[c] Titration with alcoholic KOH (16), using 3 ml. of reaction mixture.

DISCUSSION

The apparent homogeneity of the purified proteinase with respect to electrophoresis is not in itself proof that a single chemical entity has been isolated. Possible presumptive evidence for chemical homogeneity is provided by 3 properties which distinguish it from most proteins: it

contains very little tyrosine or tryptophan, it gives no positive test for sulfhydryl groups, and its electrophoretic mobility is increased, rather than decreased, by increasing ionic strength. The presence of closely related proteins with the same characteristics, however, is not excluded.

A striking characteristic of the enzyme is the dual nature of its activity. Without activator it can hydrolyze the substrate to a limited extent only, and further hydrolysis cannot be induced except by the addition of an activator such as $Fe^{++}-SH$. The ratio of amino to carboxyl groups liberated from clupein suggests that the initial enzyme may open imino linkages only; this, however, may be coincidence.

The activation of $Fe^{++}-SH$ produces both an increase in the velocity of hydrolysis and an alteration or extension of the specificity. The mechanism of activation, which is independent of any activation time (1), appears to depend upon the formation by the iron and cysteine of an addition compound with the enzyme. Fruton and Bergmann (18) have proposed a similar mechanism for the activation of papain, and have suggested that the specificities of the various activator-enzyme compounds might be expected to differ, depending upon the nature of the activator applied. Combination with foreign ions (8, 19), especially polyvalent or complex ions, may alter the mobility and shift the isoelectric point of a protein. It is possible, therefore, that the $Fe^{++}-SH$ complex may influence the charge and state of dissociation of the enzyme so that its affinity for the substrate is altered and further hydrolysis is induced.

SUMMARY

By a combination of precipitation procedures and electrophoresis, *Cl. histolyticum* proteinase has been obtained as an apparently homogeneous protein. It gives no positive reaction for sulfhydryl or disulfide groups, contains very little tyrosine or tryptophan, and, like pepsin, exhibits increased electrophoretic mobility in solutions of increased ionic strength.

Measurements of the extent to which gelatin and clupein are hydrolyzed by the enzyme before and after addition of the $Fe^{++}-SH$ activator gave evidence that a change in specificity is the basis for the increased activity of the enzyme in the presence of the activator.

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The Isolation and Identification of an Antibiotic Substance Present in the Mycelium of *Penicillium crustosum* (Thom) [1]

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INTRODUCTION

In 1944 Miller and Rekate (1) reported preliminary experiments with a mold which showed antibiotic activity against *Mycobacterium tuberculosis*. The presence of the antibiotic substance appeared to be limited to the mycelia of the mold, tentatively identified as a *Penicillium*. This finding has been substantiated and the mold classified as a strain of *Penicillium crustosum* (Thom).[3] Fermentation fluid from mold cultures grown on penicillin medium exhibited no activity against *Staphylococcus aureus* and it was believed that the active substance was not identical with penicillin. Although no activity was evidenced against *Staphylococcus aureus*, in each of a number of experiments it was shown that incubation of suspensions of tubercle bacilli with suspensions of the *mold mycelia* resulted in death of the acid-fast organisms as measured by subculture on suitable media and in some experiments by inoculation into guinea pigs.

These studies were extended to include a reexamination of the antibiotic activity of the fermentation medium as well as of the mycelia, and finally the concentration and isolation of the active agent from the mycelia. This report sets forth the pertinent detail which led to the isolation and identification of the major active component.

Experimental

Culture of P. crustosum

The first studies of *P. crustosum* were carried out on mycelia grown on Petrik's medium (2). Further work was greatly facilitated by substitution of a liquid medium for the solid medium. Such a liquid medium was prepared by simply omitting the egg protein and dye from the preparation of Petrik's medium; it consists, thus, of a fresh potato infusion made with a 12 vol.-% aqueous solution of glycerol. The medium contains, in addition, 0.1% potassium dihydrogen phosphate.

This medium may be enriched to considerably enhance the growth of the mold by the addition of asparagine, sodium nitrate or Czapek-Dox salts. A solution of 3% cornsteep solids[4] in 12% aqueous glycerol likewise forms a suitable medium.

Quantities of mycelia sufficient for isolation have been obtained by planting spore cultures from solid media on 200 cc. of potato-glycerol medium contained in each of the desired number of "glassbake penicillin culture bottles." Six to eight day surface growths at room temperature (24°–26°C.) have proven adequate as source material for isolation. Mycelia containing some activity have been obtained from "shake flask" cultures, but this method of culture has not been used as a source of mycelia for experiments on isolation. The work described below was performed on surface cultures of mycelia grown on potato-glycerol medium.

Testing of Activity

Bactericidal Activity. To a series of sterile tubes were added in turn: various aliquots of the fraction or of the mycelial suspension under test, a suspension of *M. avium*, and sterile diluent medium. A suspension of organisms sufficient to give a final concentration of 1.0 mg./cc. was routinely employed. The tubes were cultured on slants of Petrik's medium after 24 and 48 hours incubation. These subcultures were incubated and read at 48 and 72 hours.

Bacteristatic Activity. To a series of sterile tubes were added various aliquots of the fraction to be tested and each was diluted to 10 cc. with a 1:5 dilution of the potato-glycerol medium, resulting in a series of tubes which contained graded concentrations of the material under test. Inocula of the test organism, *M. phlei* or *M. avium*, were floated on the surface of the medium in each of the tubes and surface growth was observed after 48 and 72 hours incubation at 37°C. Rather than devise an arbitrary unit, the activity of various fractions has been expressed as the minimal concentration (dry weight basis) which produced complete inhibition of surface growth of *M. phlei* on a 1:5 dilution of potato-glycerol medium in 72 hours.

The bacteristatic test outlined above does not indicate activity in suspensions of mycelia. On the other hand, the bactericidal test does and the latter has been employed where it has been desirable to have a measure of mycelial activity.

Three different acid-fast organisms have been employed: a virulent strain of *M. tuberculosis*, H37Rv, obtained from W. Steenken, Jr., Trudeau, N. Y.; *M. avium*, ATCC No. 7992; and *M. phlei*, an unspecified strain obtained through the courtesy of Dr. Colin M. MacLeod, New York University, College of Medicine.

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[4] Cornsteep was obtained from the Corn Products Refining Co., Argo, Ill.

Isolation of the Active Substance. It was not possible to demonstrate the appearance of antibiotic activity in the fermentation fluid, against either acid-fast organisms or against various strains of either gram positive or gram negative organisms.

No appreciable amount of active material soluble in water was liberated by grinding the mycelia in a ball mill with an abrasive. In this way the mycelial cells were destroyed as shown by microscopic examination.

Extraction of ground [5] moist mycelia with ethyl alcohol or acetone appeared to effect fairly complete extraction of the activity. Extraction of lyophilized mycelia with petroleum ether or ethyl ether also permits complete extraction. Total extraction was evidenced by the complete inactivity of aqueous suspensions of solvent-free extracted mycelia.

The solubilities of the active material in various solvents led to the development of the following scheme for isolation of the active substance.

At the time of harvest, the spent potato-glycerol fermentation medium was decanted from beneath fifty 6–7 day cultures and discarded. The moist mycelia left in each culture bottle were wet with 50 cc. of 95% alcohol to settle the spores, and then the mycelia and alcohol were transferred to a device for shredding the mycelial mat.[6] The alcoholic slurry of mycelia was allowed to stand 24 hours at room temperature and was then filtered with suction. The mycelial cake was resuspended in a like volume of fresh alcohol and allowed to stand. The process was repeated to give a third alcoholic extract.

The combined alcoholic extracts, containing appreciable water, were evaporated under reduced pressure at 40°–50°C. From each of several lots of 50 mats approximately 750 cc. of concentrate free of alcohol, with a pH of 4.7–5.0, were obtained. This fraction, when neutral and free of alcohol, showed activity by bacteristatic test in a concentration of 800–1000 mg.-%.

This concentrate, which contained a considerable amount of immiscible tarry material, was buffered to pH 7.0 with sodium hydroxide and sodium phosphate, and extracted 5–8 times with ethyl ether, using 1 vol. of ether to 3 vols. of concentrate. The activity passed into the ether solution, together with considerable quantities of inactive substances. The remaining inactive aqueous solution was discarded.

The ether-soluble fraction was exhaustively extracted with 0.5 N sodium hydroxide, until no further material appeared to be removed with the aqueous phase (no further extraction of pigments or acid-precipitable material).

The combined alkaline extracts were acidified to Congo Red with phosphoric acid and reextracted with ethyl ether using the same volumes as before. This ether-soluble acidic fraction inhibited the growth of *M. phlei* and *M. avium* in a concentration of approximately 30 mg.-%.

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[5] Hereafter the term *ground mycelia* will be used to indicate preparations obtained by ball-milling the felted mat to a consistency fine enough to permit pipetting. Under the conditions employed, this does not result in extensive destruction of the filamentous mycelial cells.

[6] A Waring type homogenizer may be employed. For lots of 50 mats we utilized a Hamilton Beach stirrer of the type employed by soda fountains, which was fitted with sharp blades attached to the bottom of the shaft of the stirrer. Large glass bottles served as containers.

The ether solution was dried over anhydrous sodium sulfate, evaporated to a thick syrup and poured into 25 volumes of petroleum ether (b.p. 40°–50°C.) with vigorous stirring. During this addition much amorphous flocculent brown material precipitated. This suspension, on filtration, yielded a bright yellow to light reddish brown filtrate, the solids of which inhibited the growth of *M. phlei* in a concentration of 10–15 mg.-%.

Preliminary experiments on this fraction indicated that the active substance could be distilled slowly in a small sublimation unit at 75–125°C. at 4 mm. pressure. Accordingly, the fraction soluble on petroleum ether extraction of 295 mats was prepared as above and submitted to molecular distillation.[7] The petroleum ether-soluble fraction, weighing 12.5 g., was dissolved in 50 g. of a constant-yield hydrocarbon mixture with a distillation range of 70°–150°C., and distilled in a 100 cc. cyclic molecular still. Table I summarizes the data obtained on this distillation.

TABLE I

Tabulation of Data from the Molecular Distillation of an Active Fraction
Soluble in Low Boiling Petroleum Ether

Fraction	Temperature	Pressure	Weight[a]	Bacteristatic titer against *M. phlei*
	°C.	μ	g.	mg.-%
1	80	250	0.031	30
2	90	200	0.058	14
3	100	165	0.255	10
4	80	12	0.128	10
5	90	12	0.436	10
6	100	12	0.941	10
7	110	12	1.602	6
8	120	12	4.280	6
Residue[b]	—	—	3.484	10

[a] Weight of concentrate after removal from constant-yield oil hydrocarbons by extraction with aqueous alkali and reextraction of the acidified alkaline extract.

[b] The amount of residue was too small and its viscosity too great to permit further cycles through the still.

The fractions from the molecular distillation, after separation from the constant yield oil, were pale yellow in color. The first 4 fractions were oils containing suspended crystalline material; the remaining fractions were low-melting oils. The residue was a black viscous fraction containing decomposition products and appreciable residual activity.

The molecular distillation appeared to achieve partial separation of less active or inactive substances.

Later, it was found that separation of the relatively volatile constituents of the fraction soluble in petroleum ether could be achieved by the usual method of low pressure distillation through a short auxiliary heated Vigreaux column.

[7] We are indebted to Dr. Norris Embree and Dr. E. S. Perry of Distillation Products, Inc., Rochester, N. Y., who arranged for, and carried out, the distillation.

On the basis of the chemical and physical properties of the best fractions, it seemed probable that we were dealing with a substance closely akin to the unsaturated fatty acids. Accordingly, a 5% solution of fractions 7 and 8 from the molecular distillation were recrystallized at −60°C. from 1:1 methyl alcohol-acetone. The filtrate contained 52% of the original material and was bacteristatic in a concentration of 4 mg.-%.

The major portion of the filtrate from the low temperature recrystallization, as well as other filtrates prepared similarly, was employed in attempts to further purify the active substance and to provide material for chemical analysis.

Little further purification was achieved by employing various methods of chromatographic separation. Chromatographing on Doucil; [8] the long term semiautomatic type of chromatograph column developed by Cassidy (3) for the separation of fatty acids on activated carbon; and modified silica gel-buffer columns as adapted from Martin and Synge (4) by Coghill and coworkers (5) [9] removed the small quantities of pigments and neutral compounds which had followed through the previous steps. No evidence of more than one separable, active component was noted.

The most active chromatograph fractions were colorless oils with a very faint fatty odor, which inhibited the growth of *M. phlei* in a concentration of approximately 4 mg.-%.

On the basis of the activity of the most active preparations and the activity present in crude extracts, it is calculated that the mycelium contains 3–6% (extracted dry weight) of the active agent. The isolation of the pure material is not quantitative, although appreciable losses were not encountered prior to distillation; the subsequent steps entail considerable loss.

Identification of the Active Substance

At this point it appeared likely that the active substance was identical with, or very similar to, an unconjugated linoleic acid, and possibly mixed with some oleic acid. Further examination appears to establish the correctness of this assumption.

Catalytic Hydrogenation to Stearic Acid. 4.85 g. of an undistilled preparation soluble in petroleum ether were adsorbed from petroleum ether on a column of Doucil. The acidic materials were eluted from the column in 43 fractions of 25 cc. each; the first 38 by 10% absolute ether in petroleum ether and the last 5 fractions by absolute ether. Only one diffuse band was recognized from the weight chromatogram. Fractions 4–9, 13–18, 31–38, and 39–40 were pooled into 4 larger fractions as indicated, and a large aliquot of each was hydrogenated catalytically in absolute ethyl alcohol using a platinum catalyst (6). The hydrogenated products were recrystallized twice from ethyl alcohol and analyzed. Data on the twice-recrystallized hydrogenation products are given in Table II.

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[8] Doucil was obtained from the W. A. Taylor Co. of Baltimore, Md. This is a preparation used in the determination of cholesterol; it was powdered and sized to 60 mesh before use.

[9] We are indebted to the National Aniline and Chemical Co., Inc., who made available their facilities for the preparation of the silica gel.

TABLE II

Catalytic Hydrogenation Products of Chromatograph Fractions

Fraction	4–9	13–18	31–38	39–40
Concentration inhibiting *M. phlei* before hydrogenation	4 mg.-%	5 mg.-%	undet.	4–5 mg.-%
Melting point of hydrogenation product[a]	69.5–70.5°C.	69.5–70.5°C.	69.5–70.5°C.[b]	69.5–70.5°C.[b]
Analyses:[c] C	76.30	76.02	76.10	75.42
H	12.84	12.79	12.06	12.89
Neut. Eq.	281.0	—	—	—

Calculated for $C_{17}H_{35}COOH$: C: 76.00, H: 12.76: Neut. Eq. 284.5.

[a] Mixed melting points with authentic stearic acid showed no depression of melting point for any of the 4 fractions.

[b] Fractions 31–38 and 39–40 after hydrogenation contained small amounts of ether-insoluble material which were removed before recrystallization from ethyl alcohol.

[c] Carbon and hydrogen analyses were performed by Dr. Carl Tiedcke, New York, N. Y.

The physical properties and analytical data shown in Table II are in essential agreement with those required for stearic acid. The appearance of small quantities of hydrogenation products, insoluble in ether, from fractions 31–40 indicates the presence of a second unsaturated substance. It is possible that the substance giving rise to the ether-insoluble material consists of partially oxidized linoleic acid. Carbon and hydrogen analyses showed C, 73.28; H, 12.01.

Neutral Equivalents. Neutral equivalents determined on a variety of unhydrogenated chromatograph fractions gave figures between 274.0 and 281.0. The best fractions had neutral equivalents very near 280. Electrometric titration in 50% alcoholic solution with 50% alcoholic sodium hydroxide gave titration curves with a single inflection and indistinguishable from those given by linoleic acid under similar conditions.

Bromination. Two chromatograph fractions, both active in a concentration of 4 mg.-%, were bromated in *n*-hexane. The insoluble bromo derivatives which separated from each on standing overnight in the ice box were recrystallized from small volumes of ether-petroleum ether. The melting points and analyses of these preparations are given in Table III.

Since the formation of ether-insoluble bromo derivatives was not observed, the presence of more than traces of linolenic acid in such preparations is unlikely. As oleic acid is comparatively less toxic to acid-fast organisms than is linoleic acid (7) and, since pure linoleic acid was found to have quantitatively the same activity as the

TABLE III
Bromo Derivatives of Chromatograph Fractions

	M.p.	Mixed m.p. with tetrabromostearic acid	Br[a]
	°C.	°C.	Per cent
Preparation I	111–114	111–115	53.71
Preparation II	114–115	114–115.5	52.23
9, 10, 12, 13-tetrabromostearic acid	115–115.5	Calc.:	53.28

[a] The analyses for bromine were performed by Dr. Carl Tiedcke, New York, N. Y.

isolated unsaturated fatty acid fractions (see below), oleic acid does not appear to be present in appreciable quantity. The major constituent would thus appear to be linoleic acid.

Comparative Biological Activity of Linoleic Acid and Mold Preparations. Linoleic acid, prepared from 9, 10, 12, 13-tetrabromostearic acid by the method of Rollett (8), gave, after distillation, a preparation of linoleic acid which was active in a concentration of 4–5 mg.-% under the conditions of the bacteristatic and bactericidal tests which we have employed. Representative data on the bacteristatic and bactericidal activity of several preparations of linoleic acid are presented in Table IV. Data are also included on the bacteristatic activity of 3 nearly pure preparations isolated from the

TABLE IV
Bacteristatic and Bactericidal Activity of Several Preparations of Linoleic Acid,
and Crude and Purified Preparations Isolated from the Mycelia

In the bacteristatic tests, Prep. A is the filtrate from low temperature recrystallization of one of the more active molecular distillates; Prep. B is one of the best fractions from silica-gel buffer partition of Prep. A. Prep. C is similar, except that it was obtained from chromatographic fractionation on a Doucil column.

The bacteristatic titers were read as having growth (+) or showing no growth (−).

Bacteristatic titers—*M. phlei*

Concentration of added fraction isolated from mycelia	Cultures of *M. phlei*, 72 hr. readings			Concentration of added linoleic acid	Cultures of *M. phlei*, 72 hr. readings	
	Prep. A	Prep. B	Prep. C		Prep. 1	Prep. 2
mg.-%				mg.-%		
4	+	−	−	2	+	+
6	−	−	−	4	−	+
8	−	−	−	6	−	−
10	−	−	−	10	−	−
Control	+	+	+	Control	+	+

TABLE IV—*Cont.*

In the bactericidal tests, growth was evaluated as: very heavy growth, contiguous colonies; $++++$; light diffuse growth; $+++$; spotted with many colonies; $++$; 1–20 colonies; $+$; no growth; $-$.

Bactericidal titers—*M. avium*

Dilution of mycelial suspension incubated with *M. avium* 48 hrs.	Reading of subcultures after 72 hrs.	Dilution of acetone extractives incubated with *M. avium* 48 hrs.	Reading of subcultures after 72 hrs.	Concentration of linoleic acid incubated with *M. avium* 48 hrs.	Reading of subcultures after 72 hrs.	
				mg.-%	Prep. 3	Prep. 4
1/40	$+$	1/40	$+++$	2.5	$++++$	
2/40	$-$	4/40	$++$	5.0	$+$	$-$
4/40	$-$	6/40	$+$	10.0	$-$	$-$
8/40	$-$	10/40	$-$	25.0	$-$	$-$
Control	$++++$	Control	$++++$	50.0	$-$	$-$
				Control	$++++$	$++++$

mycelia of the mold, as well as bactericidal titers on ground mycelia and a crude extract prepared from the same sample of mycelia. This extract represents a neutral aqueous suspension of mycelial constituents soluble in acetone. The preparation was diluted so that 1 cc. of extract was equivalent to the acetone-soluble material from 1 cc. of the mycelial suspension.

On the basis of bacteristatic titers, linoleic acid shows quantitatively the same activity as purified material isolated from the mold. The bactericidal concentration is qualitatively the same for *M. avium* as the bacteristatic concentration for *M. phlei*, under the conditions of the 2 different tests. Comparison of the bactericidal titers of a mycelial suspension with the titer of crude extractives (see also Table V) shows that ground mycelia exhibit fully as much activity as is found in crude extracts prepared from such mycelia by simple extraction at room temperature.

To attempt, however, a quantitative comparison of the activity of suspensions of mycelia and crude fractions containing much colloidal material is open to suspicion. We are not dealing with a water-soluble agent, and the physical dispersion of the active agent is not likely to be the same in the intact mycelia as in crude extracts. Since it did not appear entirely satisfactory to equate the activity shown by ground mycelia to the agent isolated from extracts, the possibility remained that another agent could be present in the mycelium. A very labile agent might have been destroyed by the preliminary extraction procedures. Evidence for such an agent was sought by comparing the activity of ground mycelia and crude extractives with linoleic acid, in the presence of either plasma, lipoxidase or calcium.

The Effect of Plasma. Plasma, or, more specifically, the albumin fraction of plasma, will protect acid-fast organisms from the toxic action of unsaturated fatty acids (7). A given concentration of plasma added to culture media will, however, only protect the acid-fast organisms against a specific maximum amount of the unsaturated acid (9).

TABLE V

The Bactericidal Activity of Ground Mycelia, a Crude Extractive and Linoleic Acid in the Presence of Various Amounts of Plasma

The crude extractive was a neutral aqueous suspension of mycelial constituents soluble in acetone. The extractive was prepared so that 1 cc. of extract = acetone-soluble material from 1 cc. of the mycelial suspension. The cultures on Petrik's medium were read and evaluated as indicated in Table IV.

72 hr. readings of *M. avium* cultures

Dilution of mycelial suspension, incubated with *M. avium* 48 hrs.	Per cent Plasma					
	0	10	20	30	40	50
1/40	+					
2.5/40	−	++++	++++			
5/40	−	++++	++++	++++	++++	
10/40	−	−	+++	++++	++++	++++
15/40	−	−	−	++	+++	++++
20/40	−	−	−	−	−	++
Control	++++	++++				++++

Dilution of acetone extractives incubated with *M. avium* 48 hrs.	Per cent Plasma					
	0	10	20	30	40	50
1/40	++++	++++				
2.5/40	++++	++++	++++	++++		
5/40	+++	++++	++++	++++		
10/40	−	+++	++++	++++		
15/40	−	−	++++	++++	++++	++++
20/40	−	−	+	++++	++++	++++
Control	++++	++++				++++

Concentration of linoleic acid, incubated with *M. avium* 48 hrs.	0	10	20	30	40	50
mg.-%						
1.0	++++	++++	++++	++++		
5.0	−	++++	++++	++++	++++	
25.0	−	++++	++++	++++	++++	
50.0	−	++	++++	++++	++++	++++
100.0	−	−	−	+++	++++	++++
200.0	−	−	−	−	+	+++
Control	++++					++++

It was considered possible that the occurrence of a second agent, in the mycelium, might be manifest by an examination of titers of ground mycelia, crude extractives and linoleic acid in the presence of plasma. The results of one of a variety of such experiments are presented in Table V.

An examination of Table V shows that the presence of plasma in the incubated suspension of bacteria and linoleic acid decreases proportionately the bactericidal activity of the linoleic acid. The bactericidal activities of both mycelia and a crude extract are similarly altered by varying the concentration of plasma.

The Effect of Soy Bean Lipoxidase. A second substance which has a high order of specificity for linoleic acid is soy bean lipoxidase (10). The effect of this enzyme on the bactericidal activities of suspensions of linoleic acid and the mycelia was examined.

Partially purified preparations of soy bean lipoxidase and lipoxidase activator were prepared according to the directions of Balls *et al.* (10). The two preparations were filtered, respectively, through Seitz and Pyrex U. F. filters before use. A mycelial suspension of two 6 day old mycelial mats was prepared, buffered to pH 6.8 with sodium phosphate and divided into two equal aliquots of 60 cc. each. The first aliquot was centrifuged and 10 cc. of the supernatant fluid (inactive) replaced with 10 cc. of a solution of activator plus lipoxidase preparation (equivalent to 20 g. of fat-free soy bean powder). The mycelia were resuspended in the supernatant plus enzyme system to give a reconstituted suspension which was titered after incubation for 48 hours. The second aliquot was retained for control titers.

TABLE VI

Bactericidal Titers of Suspensions of Mycelia and Linoleic Acid
in the Presence and Absence of Lipoxidase plus Activator

The subcultures on Petrik's Medium were read and evaluated as indicated in Table IV.

Dilution of mycelial suspension	Readings of subcultures at 72 hrs.	Dilution of mycelial suspension with lipoxidase	Readings of subcultures at 72 hrs.
1/40	+	1/40	+ + + +
2/40	−	2/40	+ + + +
6/40	−	6/40	+ + + +
15/40	−	15/40	+ +
20/40	−	20/40	+
Control	+ + + +	Control plus lipoxidase	+ + + +
Concentration of linoleic acid		Concentration of linoleic acid with lipoxidase	
mg.-%		*mg.-%*	
2.5	+ + + +	2.5	+ + + +
5.0	+	5.0	+ + + +
10.0	−	10.0	+ + + +
40.0	−	40.0	+ + + +
110.0	−	110.0	+ + + +
Control	+ + + +	Control plus lipoxidase	+ + + +

Two suspensions of linoleic acid were prepared by neutralizing sterile solutions of sodium linoleate with phosphoric acid to pH 6.8. One suspension of linoleic acid was diluted with water to give a suspension containing 200 mg.-% linoleic acid. The second suspension was diluted with a solution of activator plus lipoxidase to give a suspension containing 200 mg.-% linoleic acid plus the enzyme system. The relative concentrations of added protein in the suspension of linoleic acid and the suspension of mycelia were the same.

Titers of each of the four suspensions were set up against *M. avium* and incubated 48 hours. At this time subcultures were made on slants of Petrik's medium and the tubes read in 72 hours. The results are shown in Table VI.

It is apparent that lipoxidase almost completely reverses the bactericidal activity of linoleic acid for *M. avium*, and similarly the activity of a mycelial suspension. The enzyme system had no observable effect on the controls. On the basis of the specificity of lipoxidase for the so-called essential type of fatty acid, and the absence of such

TABLE VII

Bactericidal Titers of a Suspension of Mycelia, a Crude Extract and Linoleic Acid in the Presence and Absence of Calcium Chloride

The subcultures on Petrik's Medium were read and evaluated as indicated in Table IV.

Dilution of mycelial suspension	Readings of sub-cultures after 72 hrs.	Dilution of same mycelial suspension containing CaCl₂	Readings of sub-cultures at 72 hrs.
2/40	++++		
3/40	++		
5/40	−	5/40+0.12% CaCl₂	++++
		11/40+0.12% CaCl₂	+++
		17/40+0.12% CaCl₂	−
Dilution of acetone extractive		**Dilution of same acetone extractives with CaCl₂**	
1/40	+++		
2/40	−	2/40+0.15% CaCl₂	+++
4/40	−	4/40+0.15% CaCl₂	++
		6/40+0.15% CaCl₂	++
		10/40+0.15% CaCl₂	+
Concentration of linoleic acid		**Concentration of linoleic acid with CaCl₂**	
mg.-%			
5	−	5 mg.-%+0.12% CaCl₂	++++
10	−	10 mg.-%+0.12% CaCl₂	++++
20	−	20 mg.-%+0.12% CaCl₂	++++
50	−	50 mg.-%+0.12% CaCl₂	++++
Control	++++	Control + CaCl₂	++++

fatty acids in the waxes of *M. avium* (11), it is noteworthy, though perhaps not surprising, that the enzyme alone exerted no bactericidal effect on this organism.

The Effect of Calcium. Kodicek and Worden (12) have shown that linoleic acid has bacteristatic activity against *L. helveticus,* and further that this action can be reversed in varying degrees by a number of organic and inorganic substances. Calcium was found to be particularly effective in this regard.

The comparative bactericidal effect, for *M. avium,* of a mold suspension, a crude extract, and of linoleic acid with and without added calcium chloride, is illustrated by the data presented in Table VII.

The bactericidal action of linoleic acid against *M. avium* was found to be prevented by calcium. The antibiotic activity of mycelial suspensions was also markedly reduced. Active, impure extracts did not show such marked reversal of activity by calcium.

Discussion

Based on the identity of the isolated agent with 9, 10, 12, 13-octadecadienoic acid, a briefer scheme of isolation might be developed. The methods could also be modified to provide additional information on the nature of the other mycelial lipides present. These possibilities appeared of insufficient importance to the immediate problem to warrant further attention. It may suffice to indicate, however, that the lengthy isolation procedure actually employed gave a variety of fractions containing metabolites of neutral, phenolic or acidic nature. None of these fractions evidenced antibiotic activity due to a compound separable from linoleic acid.

Evidence for the presence of a labile agent which might have been destroyed by preliminary extraction procedures was sought by comparing biologically the activity of ground mycelia, crude extracts, and linoleic acid. The 3 experiments reported in Tables V, VI, VII concerning the effects of plasma, lipoxidase or calcium ions were selected from a large number of experiments in which an attempt was made to specifically counteract the effect of the linoleic acid known to be present.

Comparison of the counter-inhibitory effect of plasma, of lipoxidase and of calcium on the activity of ground mycelia, crude extracts, and linoleic acid, shows, in general, that these substances decrease in parallel manner the activities of the various active preparations. Perfect parallelism would not be expected in all cases. For example, it is known that a variety of organic acids interact with plasma albumin (13). Crude extracts contain a variety of inactive acidic metabolites which could compete with linoleic acid for the plasma albumin. A similar competition would be expected in the case of calcium, whose

mechanism of action is probably even less specific than that of albumin.

Since conditions resulting in antibiosis presumably involve some type of contact between organism and agent, the hypothesis may be advanced that the linoleic acid is contained principally on the mycelial surface, perhaps as a constituent of the membrane of the mycelial cell. Linoleic acid is relatively insoluble in aqueous media and was not found to diffuse in appreciable quantity into the surrounding medium; its presence on the mycelial surface would be a prerequisite to mycelial activity on this basis. Such a hypothesis is supported by the following three observations. First, that the activity of ground mycelia is reversed by plasma and by soy bean lipoxidase, presumably nondiffusible substances. Second, that destruction of the mycelial cell by grinding with an abrasive results in an apparent decrease in activity of the mycelial suspension. Third, mycelial suspensions show no activity in the bacteristatic test as contrasted to the activity shown by the bactericidal test where adequate contact between organism and mycelia is maintained.

It is perhaps to be expected that other species of molds will show comparable antibiotic action for acid-fast organisms on the basis of the same mechanism noted for *P. crustosum*. It should be of importance, therefore, in screening tests involving mycelia or crude extracts of mycelia that the possible presence of free linoleic acid (or unsaturated fatty acids in general) should be taken into account. On the basis of observations relative to the counter-inhibitory effect of plasma and particularly lipoxidase, an adequate screening procedure should not be difficult to devise.

SUMMARY

1. Linoleic acid has been isolated in substantial amounts and identified as a constituent of the mycelium of *Penicillium crustosum* (Thom), a mold, the mycelia of which had previously been shown to possess bactericidal activity against acid-fast organisms.

2. No unequivocal evidence for the presence in the mycelium of appreciable quantities of an antibiotic agent other than linoleic acid has been obtained.

3. Some evidence is presented to support the hypothesis that the linoleic acid contained in *P. crustosum* is principally in the surface of the mycelial cell.

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Isolation of Subtilin from Submerged Cultures

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INTRODUCTION

The production of subtilin by surface (1) and submerged (2) cultur-ing methods on asparagus juice media, methods (3) for assay of subtilin, and the extraction and purification (4) of the material, primarily from surface cultures, have been described. Extraction of subtilin from sur-face cultures was effectively carried out by stirring the skimmed surface pellicle in 65–70% ethyl alcohol, followed by filtration and concentra-tion of the alcohol extract. However, in submerged cultures, where the subtilin is dispersed in the whole volume of media, this technique was not feasible, due to the large volumes involved, and another method was developed.

This paper describes in detail: (a) a method suitable for the extrac-tion of subtilin from cultures produced by large-scale submerged fer-mentation of *Bacillus subtilis* or from pellicles produced by surface culturing methods, and (b) improvements in the purification proce-dures: Essentially, the process involves extraction of subtilin from the acidified culture media with *n*-butanol, and removal of subtilin from the butanol phase by evaporation of the solvent, addition of petroleum ether (5), or addition of salt (6). Fractionation of this crude material in sodium chloride solution and washing with ethyl alcohol yields a product which appears to be homogeneous as judged by fractionation procedures, analytical values, and physicochemical characteristics. Complete characterization of this purified material will be treated in subsequent publications.

Experimental

Extraction from Culture with n-Butanol[3]

The entire submerged culture is transferred from the fermentor to a stainless-steel tank provided with a sump pump for mixing. With the pump in operation, the pH is adjusted to 2 by the addition of approximately 3 N hydrochloric acid. One-half volume of n-butanol is added and the mixture is thoroughly emulsified for 10–15 minutes by means of the pump. The emulsion is allowed to stand for approximately 12 hours,[4] again emulsified for 5–10 minutes, and the two phases separated in a Sharples[5] centrifuge (Type M-T-12-16-26 AY, fitted with a No. 29 ring dam). The volume of the wet butanol extract is generally very nearly equal to the volume of dry butanol added.

The pH of the culture before extraction must be low (below 3) for efficient extraction. In 8 representative extraction runs, at pH's from 1.5 to 2.1, the yields of subtilin[6] ranged from 84% to 96% of the subtilin present in the original culture.

Separation of Subtilin from Butanol

(a) The first method used, which involved concentration *in vacuo* of the butanol extract with resultant precipitation of the subtilin from the dry butanol, destroyed about 20% of the active material and resulted in a final product of potency lower than that produced by the following superior procedure:

(b) The preferential solubility (4) of subtilin in the aqueous phase of butanol-water-organic solvent mixtures was studied. Thirty ml. of butanol extract, 15 ml. organic solvent, and 15 ml. of 1% aqueous acetic acid were shaken thoroughly, allowed to stand 45 minutes, and centrifuged. The butanol phase was then reëxtracted in a similar manner overnight. Microbiological assay of the various phases for subtilin gave the results shown in Table I. With carbon tetrachloride and chloroform, emulsions were encountered which had to be broken by centrifugation. When petroleum ether, ethyl ether, or benzene was used, the phases separated by gravity and the aqueous extract could be readily drawn off. The following procedure, based on these results, was used in most of the runs with asparagus-juice media: One-half volume of petroleum ether was added to the butanol extract and the solution was extracted 3 times,

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[3] Suggestion of the use of butanol and experiments demonstrating its feasibility were first made by Dr. J. J. Stubbs and J. A. Garibaldi of this laboratory.

[4] Since our fermentation runs are generally terminated at about 9:00 P.M., the emulsion is allowed to stand until the next morning, when operations are resumed. The period of standing for 12 hours is not essential for the extraction, and the loss of activity on standing is negligible.

[5] The mention of this product does not imply that it is endorsed or recommended by the Department of Agriculture over others of a similar nature not mentioned.

[6] Yields of subtilin given in this paper are based on microbiological assays, using as a measure the bacteriostatic effect of the sample relative to a reference standard subtilin preparation (2). The amount of subtilin in the original culture is based on the values obtained from a 70% ethyl alcohol extract.

each time with 0.1 volume of 1% aqueous acetic acid by thorough mixing and allowing the aqueous and organic-solvent phases to separate under gravity. From 85 to 90% of the activity in the butanol was recovered in the aqueous extracts. Subtilin was precipitated out of the 1% aqueous acetic acid extract by addition of sodium chloride to 10% concentration. The precipitated subtilin, which appeared in a shallow upper layer of butanol-petroleum ether, was collected by skimming off the surface layer. It was washed with petroleum ether by decantation to remove the butanol.

TABLE I

Recovery of Subtilin from Butanol

(Percentages of Original Activity in Each Phase)

Organic solvent used for separation	Petroleum ether	Ethyl ether	Carbon tetra-chloride	Benzene	Chloro-form
Phase					
Aqueous acetic acid (1%)	68	70	66	64	49
Butanol-solvent	18	8	14	11	17
Interfacial precipitate	<1	<1	4	2	> 8
Total recovery	86	78	84	77	>74

(c) Lineweaver *et al.* (6) have recently reported a third method, involving direct precipitation at pH 5 by saturation with sodium chloride. This method, now used routinely on semi-synthetic media (7), and probably satisfactory for other media as well, is essentially as follows: Excess solid salt (60 g. of NaCl/l.) is added to the buta-nol extract with stirring and the pH of the aqueous phase adjusted to 5 by addition of 1 N NaOH to the whole mixture. The mixture is stirred vigorously for 2 hours and the precipitate, which tends to collect between the two liquid phases, is separated by centrifugation in a Sharples centrifuge (Type M-T-12-16 AY) fitted with a No. 34 ring dam. The two liquid phases come off separately, leaving the precipitated subtilin, which may then be washed free of any solid NaCl with 10% NaCl solution. At this point in the process, greater than 100-fold purification has been accomplished by both procedures (b) and (c). The potencies have ranged from 100 to 200.[7] They have been higher by procedure (c). Representative activity yields, based on the activity in the original culture, follow: They are, for procedure (b), 59, 59, 63, 71, and 56%; and for procedure (c), 63, 80, 90, 85, and 67%. In comparing these potencies and yields it must be remembered that asparagus-juice medium was used in the runs purified by procedure (b), while the semi-synthetic medium was used in runs purified by procedure (c). Direct small-scale comparisons of procedures (b) and (c) on two synthetic-media runs failed to reveal a significant difference in the yields of activity.

[7] Potency is defined as the antibiotic activity per unit of dry weight, relative to a reference standard of subtilin arbitrarily given a value of 100.

Extraction of the Moist Sodium Chloride Subtilin Precipitate with Ethyl Alcohol

The moist salt cake from either method (b) or (c) is thoroughly triturated with 10 volumes of 99% ethyl alcohol. Subtilin remains largely insoluble, while from 40 to 50% of the solids go into solution. The alcohol extract is removed by centrifuging either in a batch-type angle-head centrifuge or in a Sharples centrifuge and the extraction with alcohol repeated. The relatively inactive alcohol extracts are discarded. Recoveries of subtilin activity in this step, on four representative runs, were 98, 90, 81, and 97% on the basis of the previous 10% NaCl-insoluble fraction, or 58, 53, 51, and 69% on the basis of the original culture.

Purification of Subtilin by Fractionation with Sodium Chloride in Dilute Aqueous Solution

Purification in this step is of less importance from the standpoint of increase in potency of the subtilin preparation than from the standpoint of the removal of a small amount of toxic impurities.[8] This material is less soluble than is subtilin in 0.4% sodium chloride solution at pH 4.6. The impurity is a dark-brown gummy material with an LD_{50} of 0.2 g./kg. when given subcutaneously to mice, while the soluble fraction has an LD_{50} of 2.5–3.0. Removal of the precipitate decreases the toxicity of the product approximately 3-fold and also markedly improves the physical properties of the finished product.

The alcohol-insoluble product is dissolved in distilled water to a 0.5–0.75% solution, with a pH in the range of 3.0–3.5. Sufficient solid sodium chloride to make a 0.4% solution of salt is added and the pH adjusted with 0.1% sodium hydroxide to pH 4.6. A precipitate flocculates out, which is removed by centrifugation in a batch-type centrifuge. The supernatant extract should be perfectly clear and straw yellow in color. The precipitate is emulsified again in water, the pH adjusted to 3.0–3.5 with dilute hydrochloric acid, sodium chloride is added to 0.4%, and the pH is brought back to 4.6 with sodium hydroxide. This is repeated a third time on the precipitate with one-half the volume of water. The final precipitate is a dark, somewhat gummy material which is discarded, since it regularly has been found to contain less than 5% of the total activity. On the basis of data from 13 production batches, the approximate proportions of the subtilin in the 3 successive 0.4% sodium chloride extracts are, respectively, 70, 20, and 10.

The 0.4% sodium chloride solutions containing the active material are combined, and solid sodium chloride is added to a final concentration of 10%. Separation of the precipitated subtilin from as much of the 10% sodium chloride solution as possible is accomplished by filtration with suction through hardened filter paper and by pressing the precipitate with a rubber dam firmly fastened around the top edges of the Büchner funnel.

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[8] Addition of this salt fractionation step to the process was suggested by the observation of Dr. H. H. Anderson and co-workers that saline extracts of subtilin were less toxic than the original material.

To reduce the salt concentration of the final product, the moist precipitate is weighed, emulsified in 6.5 times its weight of water, and the precipitate collected by filtration as before. (Some activity is removed in the approximately 1% sodium chloride filtrate from this step, but it can be recovered by reprecipitation with sodium chloride as described above.) The precipitate is then emulsified in 10 times its weight of absolute ethyl alcohol, filtered, and washed twice with 3–4 volumes of absolute ethyl alcohol, and finally dried *in vacuo*. Essentially no active material is lost to the alcohol solutions.

In 7 representative isolation runs, the yields of subtilin, based on the original culture, ranged from 40 to 60%, with potencies ranging from 172 to 212. Since no method of fractionation has resulted in any marked increase in potency, it appears that these preparations may be of a high degree of purity.

EVIDENCES OF PURITY OF FINAL PRODUCT

Homogeneity on Dialysis

Subtilin dialyzes through ordinary cellophane membranes. The following experiment was carried out to determine whether any fractionation of the purified subtilin preparations could be obtained due to different rates of diffusion of active and inactive molecules which might be present. Nine grams of subtilin (No. 183) were dissolved in 90 ml. of water and introduced into a cellophane tube which was then slowly rotated in 350 ml. of 1% acetic acid at a temperature of 4°C. The outside liquid was changed after various periods of dialysis and the solutions containing the diffusate were dried by lyophilization. Potencies of the various diffusates were determined and analyses were made for sulfur and nitrogen. The results are presented in Table II.

TABLE II

*Homogeneity of Purified Subtilin Preparations on
Fractionation by Dialysis*

Fraction	Time of dialysis[a]	Weight of dialyzate	Potency	Total sulfur[b]	Kjeldahl nitrogen[b]
	hrs.	*g.*		*Per cent*	*Per cent*
A	24	3.40[c]	193	4.77	16.10
B	24	2.86	192	4.81	15.87
C	48	0.84	175	4.88	15.83
D	48	0.40	190	4.78	15.78
E	72	0.20	143		
F	72	0.12	138		
Residue		0.13	30		
Total		7.95			

[a] Individual times for each successive dialyzate.

[b] Results are on an ash-free, dry-weight basis.

[c] An undetermined amount of material was lost from this dialyzate.

Analyses of the first 4 fractions, representing at least 83% of the total amount in the bag, showed no significant differences in potency nor in sulfur or nitrogen content. The nitrogen content of Fraction A is slightly higher than the other three, but, since this fraction contained 2.7% ash compared to about 0.2% ash for the others, the possibility exists that small amounts of other nitrogen-bearing compounds may have been present.

After 6 days of dialysis only about 0.5 g. remained in the bag, two-thirds of which passed through after an additional 6 days of dialysis, leaving 0.13 g., or 1.5% of the original amount, inside the bag essentially devoid of activity. If the lower potency in Fractions E and F is due to contamination with inert material, it would mean that these two fractions contained about 0.23 g. of material of 190% potency and 0.09 g. of inert material. The total amount of inert material present would be 0.22 g., or 2.5% of the original starting material. Another very likely explanation of the low potency of E and F is that they represent inactive subtilin, which was inactivated by the long period in dilute solution. On the assumption that the potency of pure subtilin is approximately 200, the results of this experiment indicate the subtilin preparation to be of a high degree of purity.

TABLE III

Fractionation of Subtilin in 1% Aqueous Solution with Sodium Chloride

Fraction	Description	Kjeldahl nitrogen	Potency/g. of nitrogen[a]
		g.	
1	0.4% NaCl soluble, batch 160	2.31	12.4 (13.6)[b]
a1	1.5% NaCl ppt. of 1	1.49	14.1
a2	1.5–3.0% NaCl ppt. of 1	0.34	14.3
a3	3.0–10.0% NaCl ppt. of 1	0.31	14.4
b1	1.5% NaCl ppt. of a1	0.75	11.9
b2	1.5–3.0% NaCl ppt. of a1	0.40	13.1
b3	3.0–10.0% NaCl ppt. of a1	0.25	14.3
c1	1% NaCl ppt. of a2	0.08	14.1
c2	1–3% NaCl ppt. of a2	0.17	14.4
c3	3–10% NaCl ppt. of a2	0.04	14.7
d1	1.5% NaCl ppt. of a3	0.04	14.3
d2	1.5–3% NaCl ppt. of a3	0.15	15.0
d3	3–10% NaCl ppt. of a3	0.09	12.9

[a] These values represent the grams of reference standard that possess an activity equivalent to the various fractions divided by the grams of nitrogen in the fraction. If the per cent nitrogen is 15.9 in all cases (see Table II), then the potency/g. ranges from 205 to 238 if we except fraction b1 (see text).

[b] The value in parenthesis is a calculated value, based on the total recovery of activity in the first fractionation.

Homogeneity on Salt Fractionation

A 0.4% sodium chloride-soluble portion of batch No. 160 was fractionated at 3°C. from a 1% aqueous solution at pH 4.4, into 3 fractions by addition of sodium chloride to 1.5, 3.0, and 10.0% concentrations. After removal of small aliquots for microbiological and nitrogen analysis, the 3 precipitated fractions were dissolved separately in distilled water and refractionated with sodium chloride at approximately the same concentrations as before. The results of the bioassays and nitrogen analyses are given in Table III.

In none of these fractionations was there any indication of the presence of any material of significantly higher potency than the original starting material. Fraction *b1* appears to be of somewhat lower potency, but no compensation is found in the remaining fractions, indicating probably a low assay value.

Homogeneity on Electrophoretic Analysis

Dialyzate B was analyzed electrophoretically,[9] giving patterns as shown in Fig. 1. Results of this study, which will be published in detail elsewhere, indicate that the samples were essentially homogeneous electrophoretically.
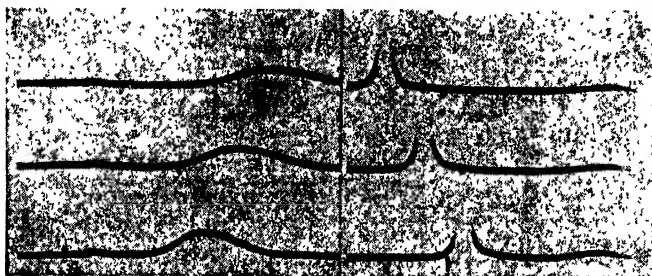
FIG. 1. Photograph showing the falling (left) and rising (right) gradients at 6020, 8000, and 10,000 seconds at 20 milliamperes, 0.62°C. Subtilin preparation 190 B in 0.099 N LiCl, HCl (about 0.001 N) to pH 2.84 (solution) or 2.90 (solvent).

DISCUSSION

As described in this paper, the isolation procedure should permit expansion to an industrial scale without too much difficulty. The direct salt precipitation (6) from the butanol extract now used routinely in semi-synthetic media production runs offers marked advantages, on a commerical basis, in simplicity and elimination of hazard over the organic solvent method used on the production runs on asparagus

[9] We are indebted to Dr. W. H. Ward of this laboratory for these results.

juice media reported in this paper and in the abstract (5). Also, under the proper conditions, filtration could replace centrifugation in some steps of the process.

Modification of the culture media would probably change the nature of the impurities that must be removed in the isolation of subtilin, and could conceivably result in a marked simplification of the purification procedure.

SUMMARY

A chemical fractionation method for the purification of subtilin from large-scale submerged cultures of *B. subtilis* has been described with manipulative details and percentage recovery at each step. The final product is obtained in 50–60% yield. It has a relative potency of about twice the reference subtilin standard used at this laboratory, and an LD_{50} for subcutaneous dosage in mice of about 3 g./kg. body weight. It appears to be essentially homogeneous as indicated by fractional dialysis, salt fractionation, and electrophoresis studies.

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The Glyceride Composition of Milk Fat

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INTRODUCTION

It is an established fact that the composition of milk fat is affected within a short period by the fat supplemented to the animals. Even though a considerable amount of work has been carried out on the composition of cow's milk fat of various countries (1–4) there seems to be no data available on the component acids of cow's milk fat from India. Since the animals are maintained in a tropical climate in India, it is of interest to know the effect of feeding oils and fats on the composition of the milk fat of such animals.

In the present study attempts were made to study the influence of supplementing a basal ration of cows with fats of different levels of unsaturation on the component acids of the resulting butter fat.

EXPERIMENTAL

Sindhi cows of about the same age, body weight and stage of lactation were selected for the feeding experiment from the Institute herd. They were divided into groups of 3 each. The normal concentrate ration of the Institute herd consisted of a mixture of wheat bran, groundnut (peanut) cake, gram[1] and gram husk in the proportion of 4.0:2.0:1.5:2.5. This mixture was fed at the rate of one pound for every 2 lbs. of milk produced by the animal. The roughage consisted of a mixture of straw (3 lbs.) and green grass (55 lbs.). The animals were kept on this ration for a week. After this control period, each animal was fed with a supplement of 0.5 lb. of the particular fat on the first day. This was increased to 1 lb. on the second day and 1.5 lbs./day thereafter, for a fortnight. The control animals were given only the normal ration during the entire period.

The groups were fed as follows:

1. Basal ration.
2. Basal ration plus 1.5 lbs. of cottonseed oil (i.v. 108.6).
3. Basal ration plus 1.5 lbs. of sesame oil (i.v. 109.4).

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[1] Bengal gram, *Cicer arietinum.*

4. Basal ration plus 1.5 lbs. of hydrogenated cocoanut oil (i.v. 4.2).
5. Basal ration plus 1.5 lbs. of hydrogenated groundnut oil (i.v. 70.4).

Butter was made from the milk collected from each group every fifth day and the iodine value of the butter fat was determined. The oil feeding was stopped after 15 days, when a day's milk was collected and the butter fat was recovered for detailed analysis. No laxative or any other bad effect was observed in the animals due to the unusually large amounts of fat ingested. The general analytical characteristics of the milk fats of the cows are presented in Table I.

TABLE I

Analytical Characteristics of Cow Milk Fat

Group	Fat supplement	B.R.[a] reading	Iodine value	Reichert value	Polenske value	Kirshner value	Saponi-fication equivalent
1	Control	41.9	33.3	27.81	1.68	24.52	245.7
2	Cottonseed oil	45.4	40.9	23.33	0.86	19.67	252.3
3	Sesame oil	45.6	44.0	23.61	1.03	19.94	254.3
4	Hydrogenated cocoanut oil	42.9	33.1	21.99	1.66	17.41	249.2
5	Hydrogenated groundnut oil	46.4	43.7	16.10	0.67	14.10	264.5

[a] Butyrorefractometer reading at 40°C.

The component fatty acids of the milk fats were determined by ester fractionation according to the method of Hilditch as modified by Smith and Dastur (7). The fat was converted into methyl esters directly and the lower component acids were fractionally separated from the whole bulk. The higher members were separated into solid and liquid acids by Twitchell's lead salt method, methylated, and also fractionally distilled. The results are shown in Table II.

DISCUSSION

The general composition of the butter fat collected from the experimental animals differs considerably from that of the butter fat from the control animals. The increase in iodine value of the milk fats from animals in Groups 2, 3, and 5 are particularly significant. Also the absence of any change in iodine value in Group 4 is worth noting. All these have a direct bearing on the composition of the fat supplemented and is in agreement with the findings of earlier workers. There is a general fall in the Reichert and Polenske values and an increase in the refractometer reading of the butter fat from the experimental

TABLE II

Summary of the Component Fatty Acids of Cow Milk Fat
Expressed in weight- and molar-%.

	Wt.-%					Molar-%				
Group	1	2	3	4	5	1	2	3	4	5
Dietary fat supplement	Nil	Cotton-seed oil	Sesame oil	Hydrogenated cocoanut oil	Hydrogenated ground nut oil	Nil	Cotton-seed oil	Sesame oil	Hydrogenated cocoanut oil	Hydrogenated groundnut oil
Butyric	3.5	3.1	3.0	2.9	2.7	9.5	8.7	8.4	8.0	7.8
Caproic	0.2	—	—	0.2	—	0.4	—	—	0.3	—
Caprylic	1.7	0.8	0.3	0.4	0.8	2.9	1.4	0.6	0.8	1.4
Capric	2.0	1.8	1.9	1.3	0.8	2.8	2.5	2.7	1.8	1.2
Lauric	1.4	2.2	1.7	5.8	1.3	1.7	2.8	2.1	7.1	1.6
Myristic	8.3	8.8	8.1	9.5	6.4	8.9	9.5	8.8	10.2	7.0
Palmitic	25.0	23.9	19.4	27.6	19.4	23.7	22.9	19.0	26.2	19.2
Stearic	16.9	12.1	14.5	12.4	15.2	14.4	10.5	12.7	10.6	13.6
Arachidic	1.5	0.6	0.8	1.1	0.9	1.1	0.5	0.7	0.9	0.8
Total	60.5	53.3	49.7	61.2	47.5	65.4	58.8	55.0	65.9	52.6
Decenoic	0.3	0.2	0.2	0.1	0.1	0.4	0.3	0.2	0.1	0.1
Dodecenoic	0.1	0.2	0.3	0.6	0.1	0.2	0.2	0.4	0.7	0.1
Tetradecenoic	0.9	1.0	0.6	1.0	0.8	1.0	1.1	0.7	1.1	0.9
Hexadecenoic	2.8	3.2	2.8	4.0	3.3	2.7	3.1	2.7	3.8	3.3
Oleic	34.2	39.9	44.4	31.0	45.3	29.4	34.7	39.4	26.8	40.6
Linoleic	0.5	0.4	—	0.4	0.2	0.4	0.4	—	0.3	0.2
C_{20-22} unsaturated	0.7	1.8	2.0	1.7	2.7	0.5	1.4	1.6	1.3	2.2
Total	39.5	46.7	50.3	38.8	52.5	34.6	41.2	45.0	34.1	47.4
Sum of the acids up to C_{14}	18.4	18.1	16.1	21.8	13.0	27.8	26.5	23.9	30.1	20.1

groups. These differences in the general composition are more clearly exemplified by the summary of results given in Table II.

From Table II it is seen that the sum of the saturated acids is 60.5% and the unsaturated acids 39.5% by weight. There exists in the literature only one detailed analysis of cow butter fat of Indian origin by Bhalerao and co-workers (in press) where the sum of the saturated acids is 61% and the unsaturated acids is 39% by weight. This agrees well with the results of the present analysis of the butter fat of the control group of cows. But there are certain differences in the distribution of the acids in the two cases. In the present case the sum of the lower acids, up to C_{14}, is 18.4, whereas it is 21.58 in the other case. There is some difference in the oleic acid content also, in spite of that sample having a higher iodine value. But this is explained by the higher linoleic acid content of the sample. These differences in the composition of the two samples may be attributed to the difference in the feed and the breed of the animals. In the present case, the feed of the animals is known, whereas in the other case the feed of the animals is not known. The fat analyzed by them is a composite sample collected from various parts of India in different seasons and from different breeds of animals. The differences in feed are therefore obvious and hence the differences in general analytical constants. Hilditch and Paul (4) and Smith and Dastur (7) have analyzed butter fat from English cows. Both of them have obtained comparatively lower values for total saturated acids compared to that of the butter fat of Indian cows. In the case of unsaturated acids, there is a considerable difference between butter fats of Indian and European origin, the amount of unsaturated acids being greater in the case of the latter. The general conclusion that can be drawn from the two sets of results is that the butter fat from Indian cows is more saturated than that of their counterparts in England. Environment, like feed, may be the chief reason, in addition to breed, for such difference.

Regarding the changes brought about in the distribution of various fatty acids in the butter fat by the ingestion of the various fats, it is more logical to compare these butter fats with that from the control animals. In the case of the control butter fat, the sum of the lower acids up to C_{14} is 18.4, whereas the ingestion of cottonseed oil reduced this only slightly to 18.1, sesame oil to 16.1, and hydrogenated groundnut oil to 13.0. But in the case of hydrogenated cocoanut oil the total amount of the lower saturated acids increased to 21.8. In studying the

effects of feeding supplements of vegetable oils to buffaloes, Anantakrishnan and co-workers (1) noticed a similar decrease of the total lower saturated acids when oils other than cocoanut were fed, and an increase of the same if cocoanut oil or hydrogenated cocoanut oil was fed as supplement. In the control butter fat the amount of the myristo-palmito-stearic group is 50.2, but this decreased considerably when any oil was ingested, with the exception only of hydrogenated cocoanut oil. Except in the case of cocoanut oil feeding the butter fats contained less of the saturated acids and more of the unsaturated acids. Here again the results with cows and buffaloes are comparable. The oleic acid of the butter fat increased when any oil was consumed by the animal, the only exception being cocoanut oil. Further, the feeding of cottonseed oil has resulted in a smaller increase of oleic acid because it contained a lower percentage of oleic acid. The linoleic acid content is not considerably affected by the feeding of the oils, but C_{20-22} unsaturated acids have found their way into the butter fat. Here again cows and buffaloes show the same adaptability. Butyric acid is characterisitc of all butter fats and, in this respect, feeding of oil supplements has, to a certain extent, reduced the amount of this acid in the butter fat. This is of particular significance in the routine analysis of butter fat for detection of adulteration as long as Reichert value is the chief criterion in judging the purity of samples.

SUMMARY

1. Cottonseed, sesame, and hydrogenated cocoanut and groundnut oils were fed to Sindhi cows at 1.5 lbs. per head per day along with basal ration.

2. The general analytical constants of the butter fat were affected by the ingestion of the oils. The Reichert value was reduced and the iodine values, butyrorefractometer readings and saponification values increased.

3. The butter fat samples collected from the experimental and control herd were subjected to detailed analysis by ester fractionation method.

4. The composition of the butter fat was considerably influenced by the oils ingested, the nature and extent of the influence depending upon the composition of the oil itself.

5. Except when hydrogenated cocoanut oil was fed, there was a

reduction in the total amount of lower acids up to C_{14}. Ingestion of cottonseed, sesame and hydrogenated groundnut oils led to an increase in the oleoglycerides of the resulting milk fat.

6. Unlike oleic acid, a high percentage of linoleic acid in the ration did not increase the amount of this constituent in the milk fat.

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A Browning Reaction Involving Copper-Proteins

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INTRODUCTION

It is currently recognized that minute amounts of metals play important roles in biological reactions, both *in vivo* and *in vitro*. It has been established that many enzymes, and other biologically active proteins, contain metals as prosthetic groups and are more or less dependent upon these metals for their activity. Inasmuch as metalloproteins have been shown to be significantly important biologically, a few examples are in order. Copper is found in living organisms as the prosthetic group of hemocyanin, polyphenol oxidase, hemocuprein and ascorbic acid oxidase; and iron is found in cytochrome oxidase, peroxidase and catalase (11). Copper and iron are not alone in representing metal constituents of enzymes. Manganese is a constituent of arginase, zinc of carboanhydrase, and aluminum of the complex succino-oxidase system in which it can be replaced by chromium (4). However, the mechanism of trace metal reactions is little understood. The literature is sketchy and, in many cases, contradictory.

In food chemistry it is a well recognized fact that off-flavors, oxidation and other deteriorations are often due to the presence of trace amounts of metals acting as catalysts. Since metals not only are present in foods in their natural state, but also may enter into the food as contaminants during compounding and processing, it is of commerical significance that a broader knowledge of their modes of action be obtained.

King (9) pointed out that copper may combine with certain proteins and react in analogous manner to ascorbic acid oxidase in the oxidation of ascorbic acid. It is possible that trace metal activity in food deterioration may be exerted mainly through reactions of this type. This

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paper will describe reactions of copper, proteins, and certain ring compounds containing an ethylene group. It will further be shown that complex end-products are formed which bear striking resemblance to some of the compounds involved in the browning reactions. In fact, it is now indicated that one type of non-enzymatic browning may be analogous to an enzymatic browning in that copper complexes with proteins, and the resulting copper-proteins act similarly to enzymes to which that metal is specific, but with deleterious effect.

Probably one of the more fundamentally sound approaches to the nature of the bonding of copper in biological materials has been made by Baudisch, who has found a simple reaction, called the B reaction, in which copper is almost unique in behaving as a central atom (2, 3, 5). This reaction is applicable to many aromatic hydrocarbons and many other ring compounds containing ethylene groups. It has also been used as a probable explanation for the activity of the copper in the polyphenol oxidases (5).

In a previous study of dry whole milk, an ideal medium for studying copper catalyzed deteriorations, it has been demonstrated that the copper is bound in the protein, that it is probably distributed uniformly in the major protein fractions, and that no electrovalent copper exists at normal pH values (15). Polarographic studies of casein containing copper indicate that the metal is strongly bound at pH 6.8–7.0.

Since work in this laboratory has strongly indicated that trace amounts of copper in foods are bound to proteins, an assumption was made that probably the mode of action might be similar to the activity of the enzyme to which the copper was specific. Assuming that the bonding of the copper might be similar to the bonding of the copper in the copper nitroso compound of the B reaction, it seemed reasonable that such copper proteins might react in an analogous manner to the cuprous salt of nitrosyl, and it was therefore decided to attempt to demonstrate complex formations between proteins which had been contaminated with copper and certain ring compounds containing ethylene groups.

EXPERIMENTAL

The first attempt was made by allowing a 1% solution of casein to react with an excess of *l*-ascorbic acid[3] in the presence of a small amount

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[3] The concentration of *l*-ascorbic acid did not appear to be critical. In this experiment the weight ratio of ascorbic acid to casein was 1 to 10. However, the reaction can be demonstrated with much more or less ascorbic acid.

(0.1 g.) of finely divided copper oxide at 30°C. and pH 6.5–7.0. No attempt was made to protect the reaction from atmospheric oxygen or light. A distinct series of color changes occurred over a period of 24 hours. The color changed rapidly from white to yellow, and finally to a dark yellow. When the reaction time was increased, the color changes proceeded through orange to a brick-red at the end of 48 hours. Adjustment of the pH to 5.0 with HCl and treatment with 2–3 volumes of absolute ethanol precipitated a reddish-brown amorphous protein substance which could be taken up with dilute NaOH and reprecipitated with acid and ethanol. The substance possessed an isoelectric point which was almost identical with that of casein, was insoluble in water or dilute mineral acids, but was soluble in dilute NaOH. Analysis showed that it contained 110 mg./g. of N_2, 9 mg./g. of Cu, a fluorescence value equivalent to 0.3 γ of quinine sulfate/g., no reduced ascorbic acid as obtained by indophenol titration, and an indicated dehydroascorbic acid activity of 200 γ/g. as determined by the Roe method (12). It was also noted that the precipitate possessed a characteristic fragrant or fruity odor.

It was not possible to obtain a reaction if any one of the 3 reacting components (copper, casein, or ascorbic acid) was omitted. However, previous treatment of the protein solution with the copper oxide, followed by filtration of the excess oxide and then adding the ascorbic acid resulted in the typical reaction. Thus, it seemed reasonably well established that the copper was bound with the protein and that the resulting copper-protein reacted with the ascorbic acid. The ability of a 1% casein solution to readily pick up copper from copper oxide at normal pH and temperature is in itself significant, in that it throws light on the ease with which foods pick up copper during processing in copper equipment.

The investigation was broadened to include the reacting of copper and casein with several compounds containing an ethylene group within a ring structure as well as certain other compounds not containing this group. These materials are listed in Table I as "reactive" or "nonreactive." It is interesting to note that compounds containing $-C=C-$ groups within a ring gave similar reactions to that described for ascorbic acid, while glucose and lactose were unreactive. Glucose, in particular, is known for its role in browning through its reaction with amino acids. This strongly indicates that the browning occurring

TABLE I

Materials Investigated for Reactivity with Copper-Proteins
(pH 6.5–7.0)

Reactive	Non-Reactive
l-Ascorbic acid	Oxalic acid
Reductic acid[a]	Glucose
Furfural	Lactose
Furfuryl alcohol	Pectin
Catechol	
Hydroquinone	
Phenol	
Pectin, acid hydrolyzed	

[a] The fluorescing properties of the browning of reductic acid are now under investigation and will be reported by Dr. T. E. Friedemann, Passavant Memorial Hospital, Chicago; trace metal activity does not come within the scope of the investigation.

with copper-casein is not the typical browning due to amine-aldehyde interaction, but may be due to a complex formation between the copper-casein and the reacting substance, in which the copper possibly acts as the central atom. The reactivity of the hydrolyzed pectin can be explained on the basis of molecular changes during the hydrolysis.

In 24 experiments reacting copper-casein with the 8 reacting substances in Table I, it was noted that there resulted no consistent or reproducible concentration of copper in the complex end-products, the copper ranging from 0.5 to 14.0 mg./g. This can undoubtedly be attributed to the fact that the protein binds copper in excess of that necessary to produce the reaction. The nitrogen contents were fairly uniform, ranging between 90 and 120 mg./g., and there was no correlation between the nitrogen and the copper contents.

All of the complex end-products formed were studied for their fluorescing properties, and polarograms from 0 to −3 volts were made on each.

Samples were dissolved in dilute NaOH and adjusted to pH 6.0 with HCl. Fluorescence values were determined on a saline solution (10), while the polarograms were made directly on the filtered solution. The fluorescence values were remarkably reproducible (within 15%) for any one of the reacting substances in Table I, but there were significant differences in fluorescence values of different reacting substances. The polarograms demonstrated two half-wave potentials which were common to all of the substances, including the unreacted copper-casein. The half-wave potentials were at −0.25 and −1.2 volts. There was a significant difference, however, for different reacting substances. For example, the ascorbic acid reaction end-product

exhibited strong waves at both half-wave potentials, while less reactive substances exhibited fairly strong waves at −0.25 volts and small waves at −1.2 volts. The unreacted copper-casein showed a fairly strong wave at −0.25 volts and a negligible wave at −1.2 volts.

To establish the order of reactivity of the 8 reacting substances with copper-casein, a controlled experiment was made in which a large amount of casein was contaminated with copper to the extent of 14 mg./g. of casein. In this particular case ionic copper was used in place of the oxide and the resulting copper-casein was blue.

TABLE II

Reactivity of Copper-Casein with Compounds Containing
an Ethylene Group in a Ring Structure

Substance added to Cu-casein	Color			Reactivity		
	During reaction		End-product	Based on color change	Based on fluorescence	Based on step height ratio
	0.5 hr.	24 hrs.	(Dry)	Visual observations	γ of quinine sulfate/g.	$\dfrac{I_{-0} \, mV}{I_{-1} \, mV}$
Reductic acid	Orange-yellow	Dark gray	Brown-black	++++	0.69	1.3
l-Ascorbic acid	Light yellow	Dark yellow	Brown	+++	0.28	1.4
Catechol	Red-brown	Red-black	Red-black	++++	0.22	1.4
Hydroquinone	Dark gray	Red-black	Red-brown	+++	0.20	1.3
Furfural	Yellow-orange	Orange-brown	Red-brown	++	0.19	1.2
Furfuryl alcohol	Yellow-orange	Orange-brown	Yellow-brown	++	0.15	1.3
Pectin, hydrolyzed	Blue	Blue	Dark blue	+	0.10	1.7
Phenol	Blue	Gray-blue	Gray-blue	+	0.05	2.2
Blank	Blue	Blue	Blue	0	Trace	2.0

(Copper oxide and copper ion can be used interchangeably with the same general results, except that the color of the casein becomes blue when sufficient of the ionic copper is used.) Identical volumes of the 1% solution were treated with the various reacting materials for exactly 24 hours at 30°C. at pH 6.6–6.8. The results of the test are presented in Table II, in which it is demonstrated that there is a definite order of reactivity of the various reacting substances as well as a correlation between the visual observations of color changes, the degree of fluorescence, and the ratio of the step height at −0.25 volts to that at −1.2 volts. Since the concentration of a given component at a given half-wave potential is a function of the current, the expression for the step height ratio is conveniently stated as $I_{-0.25v}/I_{-1.2v}$.

The blue color of the copper-casein disappeared in order of increasing activity. Thus, the blank consisting of copper-casein alone retains its blue color, while phenol and hydrolyzed pectin darken, and in all others the blue color of the cupric ion disappeared immediately and prior to any darkening. Both phenol and hydrolyzed pectin will continue to react if given a longer period of time, in which case they also yield a dark brown end-product free of the blue color with an $I_{-0.25v}/I_{-1.2v}$ of about 1.2 and 1.3. It appears that when the maximum fluorescence has developed for a given reaction the $I_{-0.25v}/I_{-1.2v}$ will be 1.2–1.4, and that a higher value indicates incomplete reactions. Although much remains to be investigated on the fluorescence and polarographic approaches, the indicated correlation between the two is certainly noteworthy.

Having established the general activity of the conjugated protein, casein, it was decided to investigate a simple protein such as albumin. It was found that, like casein, albumin entered into generally similar reactions with copper and compounds containing a $-\overset{|}{C}=\overset{|}{C}-$ group in a ring structure, yielding similar end products, but was more selective than casein. For example, copper-albumin reacted very rapidly with the aromatic compounds, but required several days to react with ascorbic acid, and then produced only small amounts of the complex end-product. Gelatin reacted similarly to albumin. Table III shows the proteins and amino acids studied and lists them as "reactive" and "unreactive."

The question of how the copper is bound in the protein still remained. If the copper were bound to free polar groups, it should be possible to replace the protein with amino acids in the reactions. A complete acid hydrolyzate of casein was selected as an ideal substitute. Neither the hydrolyzate nor the two amino acids, tryptophan and cystine in which

TABLE III
Proteins and Amino Acids Investigated
(pH 6.5–7.0)

Reactive	Non-reactive
Casein[a]	Casein hydrolyzate
Albumin[b]	(complete acid)
Gelatin	Tryptophan
Bacto-peptone	Cystine

[a] Prepared from dry milk by sodium chloride precipitation (13, 14, 15), washed repeatedly.

[b] Prepared from fresh eggs by separation, washing and filtration (6).

the hydrolyzate was deficient, were capable of replacing casein in the reactions. Thus, the postulate that the copper was bound through forces existent in polypeptides and not to amino acids was given strong support. Substitution of Bacto-peptone for protein gave analogous reactions to albumin, but this would be anticipated since peptones contain an abundance of peptides.

DISCUSSION

It seems reasonable to suggest that the reaction mechanism involved in this investigation is one in which trace amounts of copper rapidly bind to polypeptides and the resulting copper-protein combines with certain ring compounds containing ethylene groups. The possibility of such types of reactions occurring in foods or, for that matter, other biological materials which contain copper, either naturally or through contamination, is obvious on the basis of the composition of those materials. It is further suggested that trace amounts of copper bound to proteins react in an analogous manner to the enzymes to which copper is specific. Considering the specificity of such enzymes as ascorbic acid oxidase or tyrosinase, it becomes obvious that specific types of linkage are involved. On the other hand, when whole proteins are contaminated with copper the copper distribution is undoubtedly quite general. Thus, with copper-casein we might expect the quite generalized reactions obtained as contrasted to the specificity of the enzymes. It does seem reasonable, however, that different proteins would demonstrate some generalized specificity for certain groups of compounds on the basis of the difference in protein molecular structure. Thus, it is not surprising that we find copper-casein generally reactant to all the compounds

investigated, while copper-albumin is more selective. The latter reacted immediately with the aromatic compounds, but very slowly with ascorbic acid.

It is well recognized that the browning of foods is extremely complex and involves many reactions. Although it has been suspected that trace metals may catalyze browning, it was only recently that it was shown that trace amounts of metals, particularly copper, materially accelerate the rate of browning (1). The results of the present investigation show that there is probably a very marked involvement of, at least, copper.[4] The complex end-products which have been isolated all possess common characteristics of browning end-products. They range in color from dark reddish-brown to black, possess a high degree of fluorescence, and are characterized by odors which can generally be classified as fragrant, acid, burnt and caprylic in accordance to the Crocker-Henderson Test (7, 8). The complexity of the odors was well evidenced by the remarks of several testers when they examined these materials. For one of the samples they suggested "fragrant," "vanilla-like," "hint of garlic," "burnt," and "fruity." In the authors' opinion "fruity" and "caramel" odors predominated in all of the compounds except those obtained using furfural and furfuryl alcohol, which retained their typical furfural odor.

It is significant that these reactions were obtained at near room temperatures (about 30°C.) and normal pH. Although no special effort was made to study the reaction at elevated temperatures, it was noted in a few tests with copper-albumin and l-ascorbic acid that the reaction could be accelerated by elevating the temperature to 50°C.

Results of this investigation have indicated that trace amounts of copper in foods are bound to proteins or polypeptides and can result in a browning reaction which appears to be very similar to certain types of enzymatic browning. Since other metals may exert their activity in a somewhat analogous manner to copper, the significance of this type of reaction cannot be overlooked. It is necessary that enzymes must be inactivated if a processed food is to have maximum stability. However, the blanching employed to accomplish inactivation does not decrease the concentration of the metals present, and it may even increase the metal content through contamination during the process. It is possible that, in certain cases, recurrence of enzymatic activity,

[4] In naturally occurring systems the copper concentrations would be much lower than those studied in this investigation. However, it may be presumed that the diluted reactions would proceed at a slower rate.

which is usually attributed to incomplete or inadequate blanching, may in actuality be due to the presence of one or more metals in the form of metallo-proteins.

ACKNOWLEDGMENT

SUMMARY

1. Reactions of copper, proteins and certain ring compounds containing an ethylene group are described. These reactions are accompanied by marked changes in color as well as development of fragrant odors and fluorescence.

2. It is shown that proteins can readily bind copper from copper oxide, and that the copper in the active complex is probably bound through forces existent in polypeptides and not to amino acids.

3. It is indicated that copper-protein complexes can promote a browning, the mechanism of which is different from known types of non-enzymatic browning.

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Comparative Substrate Specificity Studies of Ascorbic Acid Oxidase and Copper Ion Catalysis[1]

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INTRODUCTION

The two widely recognized catalysts active in the oxidation of *l*-ascorbic acid, ionic copper, and ascorbic acid oxidase are both non-specific for this compound. Copper ion has been shown to be an effective catalyst in the oxidation of a number of biologically active compounds, including cysteine (1), glutathione (2), thioglycollic acid (3), and dihydroxymaleic acid (4).

Zilva *et al.* (5, 6) found "ascorbic acid oxidase" to be non-specific in activity and reported aerobic oxidation of *l*-ascorbic, *d*-araboascorbic, *l*-glucoascorbic and *l*-galactoascorbic acids by cucumber juice. A lack of substrate specificity is characteristic of a copper-albumin complex reported by McCarthy, Green and King (7), which acts as a catalytic agent on *l*-ascorbic and *d*-isoascorbic acid. The polyphenol oxidase of Kubowitz (8), also a copper-containing enzyme, while ineffective on ascorbic acid directly catalyzes the oxidation of a number of *o*-diphenol type compounds. The copper protein oxidases, while shown to be nonspecific for a single compound, do exhibit specificity for a type structure. These demonstrations gain new interest because of the antibiotic activity of *d*-glucoascorbic acid reported by Woolley (9).

The theory of analogue substitution for an essential metabolite at a specific site in an enzymatic system has been an attractive and fruitful one in antivitamin research. Ascorbic acid oxidase provides an accessible enzymatic system which may exhibit substrate specificity based on chemical structure. Such a study is reported using as substrates analogues of *l*-ascorbic acid and compounds which have structurally similar

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chemical groupings. A simultaneous study of ionic copper with the same substrates served to compare the two catalytic systems.

Experimental

The partially purified ascorbic acid oxidase used in this study was made from cucumber press juice. The pH of the press juice was 5.6–5.7. It was raised to pH 7.5 by means of 10% sodium carbonate and the green cellular material which separated was discarded. The supernatant liquid was made 0.75 saturated with dry ammonium sulfate, chilled and filtered. The precipitate was taken up in water with the aid of 0.1 M disodium phosphate to complete the dispersion. The pH of this suspension was 6.9. It was lowered to pH 5.6 by the addition of 10% acetic acid and made 0.5 saturated at room temperature by the addition of an equal volume of saturated ammonium sulfate. The precipitate which formed was dispersed in water and allowed to stand 12 hours at refrigerator temperature. The precipitate which formed at this temperature was discarded. Tenth-molar disodium phosphate was added to pH 7.0, the preparation warmed in a water bath to 37°C., and the material which separated discarded. The pH of the solution was again lowered to 5.7, chilled and made 0.5 saturated by slowly adding an equal volume of saturated ammonium sulfate at 10°C. After standing for 30 minutes the precipitate was centrifuged. It readily dispersed in water and was water white. Overnight standing in a refrigerator resulted in the settling of a precipitate. This was discarded and the supernatant used to accumulate the data presented. Denaturation at this point was marked, and further efforts to fractionate resulted in rapid loss of enzymatic activity. The preparation used had a copper content of 0.028% and an oxygen uptake of 15,000 mm.³/mg. of enzyme (dry weight)/hr. with l-ascorbic acid as substrate.
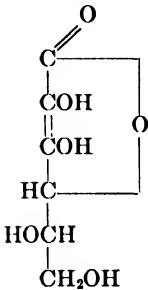
The fractions used to follow the preparation were dialyzed against water triple-distilled in glass, dried in weighing vials to constant weight, wet ashed by sulfuric and perchloric acids and tested for copper by the dithizone method of Fischer and Leopoldi (10). This method was adapted for use in the Evelyn photoelectric colorimeter.

If this preparation is referred to the highly pruified ascorbic acid oxidase of Powers, Lewis and Dawson (11) and the comparison based on its copper content, it can be judged to be in the range of 10% purity; if based on the oxygen consumed, the purity would be judged to be only about 2.5%, possibly due to the denaturation of the preparation.

The activity of both catalysts was measured manometrically in air-filled Warburg vessels of the Erlenmeyer type, at a temperature of 37.2°C. The concentration of all substrates was 1×10^{-2} mM in 3.3 ml., the total volume of the solution in the vessels. For the enzyme series the solution was buffered to pH 5.7 ± 0.1 by phosphate buffer. In the copper catalysis 1×10^{-4} mM copper ion was added and data in the range pH 5.4–5.9 are presented. They are taken from a study covering a wide pH range, with a change in buffer systems from phthalate at pH 5.4 to phosphate at pH 5.9. For both buffer systems the oxygen uptake was corrected for by blanks. The rate of oxygen uptake was established by using that part of the cruve which was linear. For the copper series at this concentration of substrate and catalyst this was the first 15 mins. For the enzyme series it was based on agreement in oxygen uptake in the first 4

TABLE I

Summary of Enzyme and Copper Catalysis of Oxidation

Substrate	Enzyme oxidation oxidase—0.028% copper pH 5.7	Copper oxidation copper ion—1×10^{-4} mM pH 5.4–5.9
	Oxygen uptake mm.^{3}/mg./hr.	Oxygen uptake mm.^{3}/copper added/hr.
l-Ascorbic acid	15,000	400

```
      O
      ‖
      C
      |
      COH
      ‖      O
      COH
      |
      HC
      |
     HOCH
      |
     CH₂OH
```

d-Isoascorbic acid	12,750	450

```
      O
      ‖
      C
      |
      COH
      ‖      O
      COH
      |
      HC
      |
     HCOH
      |
     CH₂OH
```
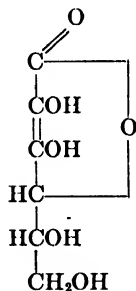
d-Glucoascorbic acid	4,900	450
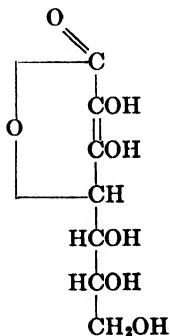
```
   O
    ‖
    C
    |
    COH
    ‖
    COH
    |
    CH
    |
   HCOH
    |
   HCOH
    |
   CH₂OH
```

TABLE I (*continued*)

Substrate	Enzyme oxidation oxidase—0.028% copper pH 5.7 Oxygen uptake mm.³/mg./hr.	Copper oxidation copper ion—1×10⁻⁴ mM pH 5.4–5.9 Oxygen uptake mm.³/copper added/hr.
Imido d-Glucohepto-ascorbic acid	None	275
Reductinic acid	6,675	375
Dihydroxymaleic acid	None	230
Dihydroxyacetone	None	None
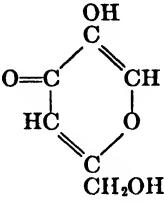
Imido d-Glucohepto-ascorbic acid

```
     NH
    ∥
    C────┐
    ‖    │
    COH  │
    ‖    O
    COH  │
    │    │
    HC───┘
    │
  HOCH
    │
  HCOH
    │
  HCOH
    │
  CH₂OH
```

Reductinic acid

```
      O
     ∥
     C────┐
     │    │
     COH  │
     ‖    │
     COH  │
     │    │
    CH₂───CH₂
```

Dihydroxymaleic acid

```
   COOH
    │
   COH
    ‖
   COH
    │
   COOH
```

Dihydroxyacetone

```
   CH₂OH
    │
    C=O
    │
   CH₂OH
```
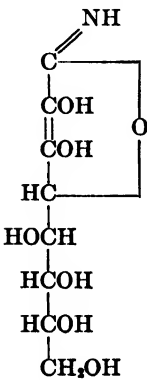
TABLE I (*continued*)

Substrate	Enzyme oxidation oxidase—0.028% copper pH 5.7 Oxygen uptake mm.³/mg.,hr.	Copper oxidation copper ion—1×10⁻⁴ mM pH 5.4–5.9 Oxygen uptake mm.³/copper added/hr.
Kojic acid	None	None
Mannosaccharic acid	None	Slight autoxidation
Glucuronic acid	None	None
Hydroquinone	None	Slight autoxidation

Kojic acid

OH
C
O=C   CH
HC   O
C
CH₂OH

Mannosaccharic acid

COOH
HOCH
HOCH
HCOH
HCOH
COOH

Glucuronic acid

CHOH
HCOH
HOCH
HCOH   O
HC
COOH

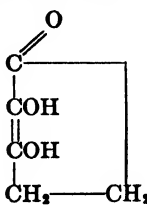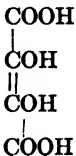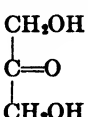Hydroquinone

OH
C
HC   CH
HC   CH
C
OH

TABLE I (*continued*)

Substrate	Enzyme oxidation oxidase—0.028% copper pH 5.7 Oxygen uptake mm.³/mg./hr.	Copper oxidation copper ion—1 ×10⁻⁴ mM pH 5.4–5.9 Oxygen uptake mm.³/copper added/hr.
Catechol	None	Slight autoxidation

minutes at two dilutions of the preparation. The substrates were: *l*-ascorbic, *d*-iso-ascorbic, *d*-glucoascorbic, imido *d*-glucoheptoascorbic,[3] reductinic,[3] dihydroxymaleic, kojic, mannosaccharic and glucuronic acids; dihydroxyacetone, hydroxyquinone and catechol (Table I).
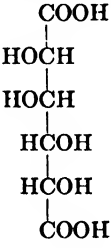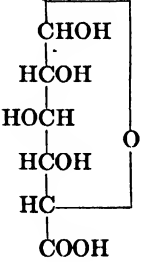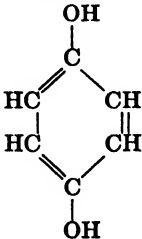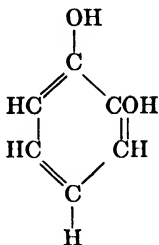
RESULTS

The enzymatic catalysis with *l*-ascorbic acid as substrate was always characterized by an oxygen uptake which was theoretical for oxidation to the reversible or dehydro form of the acid. For the copper ion catalysis of *l*-ascorbic acid the oxidation resulted in an oxygen uptake always greater than one atom per molecule of substrate. This distinguishing feature of the two types of catalysis has been noted by Hand and Greisen (12) and Steinman and Dawson (13).

The catalytic action of the enzyme was approximately the same for *l*-ascorbic acid and its six-carbon isomer *d*-isoascorbic acid. For the seven-carbon analogue, *d*-glucoascorbic acid, the rate was decreased to about one-third. The eight-carbon compound, imido *d*-glucohepto-ascorbic acid, in which the oxygen of the first carbon is replaced by an imido group, showed no oxidation in the presence of the enzyme. The oxidation of reductinic acid, in which the first three carbons are similar to those of the ascorbic acid analogues, was catalyzed by the enzyme. Dihydroxymaleic acid, which has the dienol group but no ring structure, was not catalyzed by the enzyme. Kojic, mannosaccharic and

[3] The reductinic and imido *d*-glucoheptoascorbic acids were furnished by T. Reichstein.

glucuronic acids, hydroquinone, catechol and dihydroxyacetone were not oxidized in the presence of the ascorbic acid oxidase preparation.

The specificity exhibited by the enzyme is apparently for those compounds whose structures contain a dienol grouping adjacent to a carbonyl group and closed ring structure. This point is illustrated especially well by the inability of the enzyme to catalyze the oxidation of imido d-glucoheptoascorbic acid and dihydroxymaleic acid. Both compounds have the dienol grouping, but the first carbon is altered by the substitution of an imido group for oxygen in one case, and by the lack of opportunity to form a lactone bridge (and so a carbonyl group) in the second case.

With cupric ion the oxidation of the ascorbic acid series, l-ascorbic, d-araboascorbic, d-glucoascorbic and imido d-glucoheptoascorbic acids was readily catalyzed. Reductinic acid, whose first three carbons have the same structure as the ascorbic acid analogues, was oxidized, but so was dihydroxymaleic acid, which shares only the dienol grouping. Dihydroxyacetone was not affected, nor was kojic acid, which has a structure including double bond, ketone and hydroxyl groups. Glucuronic and mannosaccharic acids showed no catalyzed oxidation by copper ion. Hydroquinone and catechol showed only a slight amount of autoxidation.

SUMMARY

Two catalytic agents, a preparation of the enzyme ascorbic acid oxidase from cucumbers and copper ion, have been compared on the basis of their activity on the oxidation of l-ascorbic acid and a series of compounds which have structural similarities.

Within the scope of this series of substrates, the enzyme catalyzed only the oxidation of those compounds which have the dienol group adjacent to a carbonyl group and ring structure. It was ineffective for the dienol alone. The enzyme was further characterized by oxidation of the compounds of the ascorbic acid series only to the reversible stage.

The effect of copper ion on the oxidation of the same substrates indicated that, under the conditions maintained in this study, it was specific as a catalyst for the dienol group. This catalyzed oxidation of the ascorbic acid series was characterized by an oxygen uptake greater than theoretical for the change to the reversible stage.

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Changes in Ascorbic Acid Content of Turnip-Leaf Discs as Influenced by Light, Temperature, and Carbon Dioxide Concentration

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INTRODUCTION

Previous work (2, 3, 6, 7, 9, 12, 17) has shown that light plays an important role in determining the ascorbic acid content of many plant tissues. However, this apparently is not true of all plant tissues. It is well known, for example, that ascorbic acid is formed during the germination of various seeds in the dark (see 9, 13, 15, and 18 for some recent data). Similarly, Smith and Walker (16) have concluded that for cabbage there is no simple relationship between ascorbic acid content and day length or the light energy over long periods. This indicates that the relationship of light to ascorbic acid formation needs further study, especially since there has been no indication, to date, of the mechanism whereby light may influence the ascorbic acid content of plant tissues.

In previous studies, either more or less complete plants or whole leaves were used. The use of intact plants, or even whole leaves, puts certain limitations upon experiments of this kind. One such limitation results from the great variation in the ascorbic acid content of the different tissues. Even similar tissues, which are comparable in many other respects, may differ widely in their ascorbic acid content. For example, leaves of the same age from different plants which have been grown under as nearly identical conditions as possible usually differ somewhat in their ascorbic acid content. As a consequence, large numbers of plants must ordinarily be used so that a satisfactory sample can be obtained. To study the influence of such factors as temperature, light, and carbon dioxide concentration under controlled conditions with large numbers of whole plants requires rather elaborate facilities. Hence, it seemed desirable to use a simpler technique.

In the present paper the application of such a technique will be presented, along with some preliminary results which have been

obtained. Results of more extensive studies will be published subsequently.

MATERIALS AND METHODS

Turnips of the Shogoin variety were grown in sand culture using Hoagland's nutrient solution No. 1 supplemented with micronutrient elements (4). The plants were grown in the greenhouse until they produced leaves at least 20–25 cm. long. In each experiment, all of the leaves used were of a comparable morphological age, *i.e.*, leaves just below the youngest fully expanded leaves. Only one or two leaves were used from each plant in any one experiment. The preparation of the individual samples can be illustrated by giving the details for a single experiment. With inconsequential changes, these details apply to all of the experiments.

In Experiment No. 9, ten discs, each 1.6 cm. in diameter, were cut from each side of the midrib of 15 leaves with a stainless steel cork borer. One disc (at random) from each half of each leaf was placed on moist filter paper in a covered petri dish. Thus there were 10 petri dishes, each of which contained 30 discs; and the contents of each petri dish constituted a single sample. Duplicate samples were floated on Hoagland's solution as described below and then placed in the dark in constant-temperature chambers at: 10°, 19°, and 30°C. Two more samples were analyzed immediately, and the remaining two samples were given other treatments, the results of which are not reported.

Before the samples were placed in the dark, they were transferred to large crystallizing dishes (19 cm. diam.) or similar containers, where they were floated on Hoagland's solution. Each disc was floated with the bottom (abaxial) side up to allow as much opportunity as possible for gaseous exchange through the stomates. The dishes containing the discs were then submitted to the various environmental conditions, and samples were removed for analysis after 48 hours. The results are presented in terms of γ of ascorbic acid per disc in all cases. The fresh weights of the individual discs or samples were not determined, but other similar discs from turnip leaves weighed about 30 mg. each.

The analytical method used was the indophenol-xylene extraction method described by Nelson and Somers (10). Each sample was divided into lots of 15 discs for each analysis. Each lot was blotted with filter paper and was ground in a Waring blendor with 50 ml. of 3% metaphosphoric acid solution in a small bowl. Two or more aliquots of the filtrate from each lot were analyzed. The order of magnitude of the sampling error is indicated by the following: Two samples (from another experiment), each composed of 3 lots of 15 discs each, were analyzed immediately after being cut from the plant. The means and corresponding standard errors for the two samples were 44.0 ± 1.20 and 45.4 ± 1.46 γ of ascorbic acid per disc, respectively. In still another experiment, 4 samples, each composed of two lots of 15 discs each, were analyzed after being exposed to artificial illumination for 48 hours. The mean of all 8 lots of discs and the standard error were 90.6 ± 2.33 γ of ascorbic acid per disc.

In all cases, the source of illumination was fluorescent lights. "Daylight" and "white" fluorescent tubes were used in equal proportions. The control of the temperature of the solution upon which the discs were floated presented a problem. Stirring of this solution was found to be undesirable since this caused the discs to group together

rather than remain uniformly distributed over the surface. It also resulted in the submergence of some of the discs which had to be avoided to obtain satisfactory gaseous exchange. The procedure finally used was to place the dishes containing the floating discs in rooms or incubators in which the air temperature was maintained constant. At temperatures below 25°C., this gave fairly good control. The fluctuations in air temperature were ordinarily not in excess of ±0.5°C. With temperatures over 30°C., it was found more difficult to control the air temperature, and the fluctuations were usually about ±1.0°C. In all experiments, the temperature of the nutrient solution was measured frequently with thermometers.

In experiments in which the concentration of carbon dioxide in the air was varied, each dish containing the floating discs was supported in a large glass desiccator which was closed with a glass plate. In the bottom of the desiccator was placed 500 ml. of 0.2 N H_2SO_4 into which the required volume of 1.0 M $NaHCO_3$ was tipped after the desiccator had been closed. This caused a small increase in pressure inside the desiccator, but no evidence of escaping gas was detected. The discs were floated on Hoagland's solution (pH about 5). The free gas space was 6500 ml. In those cases in which a carbon dioxide-free atmosphere was used, the sulfuric acid was replaced with 500 ml. of a 5% NaOH solution.

RESULTS

Influence of Temperature in the Dark

The data in Table I (Expts. 9 and 10) summarize results obtained when the rate of ascorbic acid disappearance in the dark was measured at various temperatures. Each of the values for ascorbic acid content represents the mean of two samples, each composed of duplicate lots

TABLE I

The Influence of Temperature upon the Rate of Change in Ascorbic Acid Content of Turnip Leaf-Discs

| Experiment number | Duration of exp. | Initial ascorbic acid content[a] | Change in ascorbic acid content at various temperatures | | | | | | Conditions |
			Temp.	Ascorbic acid[a]	Temp.	Ascorbic acid[a]	Temp.	Ascorbic acid[a]	
	Hrs.		*°C.*		*°C.*		*°C.*		
9	48	47.6	10.5	− 3.7	19.0	−12.9	29.0	−24.7	Dark
10	47	58.6	10.5	−10.6	19.0	−22.1	30.5	−31.2	Dark
11	19	64.0	14.3	+ 5.4	23.0	+15.2	28.2	+22.3	1400 f.c.[b]
12	47	59.0	14.1	+22.7	24.0	+43.2	29.0	+45.0	1400 f.c.
13	47	64.0	14.7	+18.9	24.0	+42.0	30.5	+45.4	1400 f.c.

[a] γ/disc.

[b] f.c. = the amount of illumination, in foot-candles, as measured by a Weston Sunlight Illumination Meter (Model No. 603).

of 15 discs. In all cases the amount of ascorbic acid decreased in the dark. Temperature coefficients calculated from these data range from 1.4 to 4.3. These values are subject to considerable experimental error, but they indicate that the overall rate is determined by ordinary thermal reactions.

Influence of Temperature in the Light

The data in Table I (Expts. 11 to 13) summarize the results obtained when the rate of ascorbic acid accumulation in illuminated discs was measured at various temperatures. Each of the values for ascorbic acid in Expts. 12 and 13 represent the mean of two samples, each composed of duplicate lots of 15 discs. In Expt. 11 only single samples, each composed of duplicate lots of 15 discs, were analyzed for each value given in the table. The light intensity (as measured by a Weston Sunlight Illumination Meter, Model No. 603) was about 1400 foot-candles in all 3 experiments. The temperature control was not very good, particularly at the highest temperature. Nevertheless, the results are indicative of the influence of temperature. They indicate that the temperature coefficient in the light over the lower temperature range is greater than over the higher temperature range; the average values for Q_{10} are 2.5 and 1.4, respectively.

Influence of Carbon Dioxide Concentration

The data in Table II summarize the results of two experiments in which the rate of ascorbic acid accumulation was measured in the light in the presence or absence of carbon dioxide. The value given for carbon dioxide concentration is the approximate concentration at the

TABLE II

Influence of the Presence or Absence of Carbon Dioxide upon the Changes in the Ascorbic Acid Content of Turnip Leaf-Discs in the Light

Experiment number	Duration of exp.	Temperature	Initial ascorbic acid content[a]	Change in ascorbic acid content[a]		Light
	Hrs.	*°C.*		*0% CO_2*	*5% CO_2*	*Foot-candles*
2–19	45	31	49.5	−32.0	+84.4	1200
2–21	47	32	48.4	−36.2	+94.7	1200

[a] Ascorbic acid content is expressed as γ/disc.

beginning of the experiment. Each value for ascorbic acid content in Expt. 2–19 represents the mean of duplicate lots of 15 discs, whereas in Expt. 2–21 these values are the means of triplicate lots of 15 discs each. It is evident that the accumulation of ascorbic acid in turnip leaf discs requires the presence of carbon dioxide.

It will be noted that in the absence of carbon dioxide there is a decrease in ascorbic acid concentration. This result has been found consistently throughout a number of experiments, even though there is some variation in the magnitude of the effect. Similar results are obtained when the effect of two air streams, one moist and CO_2-free and one simply moistened, are compared in the light at the same temperature. In some experiments, the decrease in ascorbic acid in the light in the absence of carbon dioxide is greater than the decrease in air in the dark at the same temperature.

TABLE III

The Influence of Light Intensity upon the Accumulation of Ascorbic Acid in Discs from Leaves of Various Plants

Plant	Initial ascorbic acid content[a]	Change in ascorbic acid content[a] at various light intensities				Av. weight of a disc
		Dark	200 f.c.[b]	500 f.c.[b]	1200 f.c.[b]	
						mg.
Tomato	24.4	−19.4	− 9.6	—	− 6.7	28
Bean	39.4	−38.9	− 2.4	− 4.3	+ 3.6	37
Brussels sprouts	120.1	−17.2	− 2.7	+25.9	+42.2	54
Broccoli	106.6	−23.2	+13.3	+34.4	+58.6	54

[a] γ/disc.

[b] f.c. = the amount of illumination, in foot-candles, as measured by a Weston Sunlight Illumination Meter (Model No. 603).

The temperatures in this case were 22°–23°C. in the dark and at 200 f.c., 24°–25°C. at 500 f.c., and about 26°C. at 1200 f.c.

This same technique has been applied to discs cut from leaves of other plants. The data are summarized in Table III. In the case of discs cut from tomato leaves grown in the sun, there is no increase in ascorbic acid content in 45 hours at intensities up to about 1200 foot-candles. However, the rate of disappearance of ascorbic acid is influenced by the light intensity, at least up to 1200 foot-candles. As seen in Table III, similar results were obtained with discs cut from leaves of

field-grown yellow snap beans. With discs cut from field-grown Brussels sprouts leaves, there was an accumulation of ascorbic acid in 45 hours at 500 and 1200 foot-candles. At 200 foot-candles there was no significant change in ascrobic acid content, but in the dark there was a loss in ascorbic acid. Somewhat similar results were obtained with discs cut from leaves of field-grown broccoli, except in this case a significant accumulation occurred with a light intensity of 200 foot-candles. Thus it appears that the response of discs from various plants differs considerably, but in all cases the ascorbic acid changes are influenced by light intensity. There were some differences in temperature between the various treatments (see Table III footnote), but these differences were not large enough to vitiate this conclusion.

In all of the experiments reported above, the discs were floated for 48 hours or less before being analyzed. Other experiments have been conducted in which the discs were floated for as long as 96 hours exposed to air in a large room. In the light, the ascorbic acid content continues to increase throughout this period. However, shorter periods were used in the above experiments because sufficient changes were obtained to make the analyses significant, and because there is some tendency for the discs to die and become infected with fungi when they are floated for 4 days.

DISCUSSION

It is surprising that as low a light intensity as 500 foot-candles, even though it is continuous, would result in an accumulation of ascorbic acid in discs cut from leaves of field-grown plants. The intensity of full sunlight in Ithaca at the time these studies were made is about 10,000 foot-candles. Possibly ascorbic acid is translocated from leaves to other parts of the plant. In the case of the discs, this cause of a decrease in ascorbic acid content probably would be greatly reduced, if not eliminated entirely. Some ascorbic acid may diffuse from the discs to the suspending liquid. Due to the instability of ascorbic acid in this solution, such a loss would be difficult to measure accurately. However, the discs floated *on* the solution; hence there was little contact of the cut vascular tissue with the nutrient solution, and probably the amount of ascorbic acid lost by diffusion was not significant. It may be that the data obtained by using discs give some measure of the capacity of leaf tissue to produce ascorbic acid, and the ascorbic acid content found in

intact plants is the resultant of the balance between this capacity and the rate of utilization of ascorbic acid by the plant.

So far as the authors are aware, these studies are the first in which discs of leaf tissue have been used to study ascorbic acid changes, although leaf discs have long been used in other physiological studies, principally for analytical purposes. The above results indicate that such discs behave, with respect to changes in ascorbic acid content, in much the same way as intact plants.

Some studies of this kind have been carried out with whole leaves. Wolf (19) measured the ascorbic acid changes in detached leaves of *Bryophyllum calycinum* in the dark at various temperatures. He found little or no change in 43 hours at 7° and 37°C., but at 20°C. he observed a rapid decrease. On the other hand, we found a decrease in ascorbic acid content at all of the temperatures we used. The reason for the apparent disagreement between the two sets of data is not clear, but one of us (G. F. S.), in unpublished experiments, has observed that detached leaves of *Bryophyllum Daigremontianum*, a species closely related to that used by Wolf, wilt more readily at 20°C. than at 13° or 31°C. Whether or not such a phenomenon is related to the ascorbic changes observed by Wolf is not known. Moldtmann (9) used detached fig leaves to study changes in ascorbic acid content under various conditions. He found that both carbon dioxide and light were essential.

Temperature influences the rate of ascorbic acid loss in seedlings and some vegetables in the dark in much the same way as reported above for turnip leaf discs (11, 13).

Weissenböck and Weissenböck (18) grew plants in normal air and in air from which carbon dioxide was removed. With seedlings, the absence of carbon dioxide produced no differences in ascorbic acid content over periods of 6–10 days. With older plants, periods of 2–3 weeks in the absence of carbon dioxide resulted in a lower ascorbic acid content. Whether or not the results of such prolonged treatments are directly comparable with results cited above will have to await further research. It seems probable that such prolonged treatments may have produced more drastic changes in the plant tissues than were produced by the relatively short periods used in the above experiments.

It is reasonable to assume that photosynthesis occurred in the illuminated discs in these experiments, particularly since other studies have shown that photosynthesis occurs in discs cut from spinach leaves

(1). It is rather interesting to note the similarity between the influence of various factors upon ascorbic acid accumulation and photosynthesis (see also Moldtmann, 9). It is well known that photosynthesis requires light and carbon dioxide, as does ascorbic acid accumulation. Our data on the influence of temperature upon ascorbic acid accumulation in the light show some similarity to the well-known influence of temperature upon photosynthesis. However, the experimental errors involved in our measurements of Q_{10} are so great that this apparent similarity of the influence of temperature on ascorbic acid accumulation and photosynthesis should be considered only as a preliminary indication.

SUMMARY

1. Discs cut from leaves of turnips and other plants have been used to study the influence of various factors upon the rate of change in ascorbic acid content of leaf tissues. These discs are floated on a mineral nutrient solution and exposed to various environmental conditions before being analyzed.

2. The rate of decrease in ascorbic acid content of turnip leaf discs floated in the dark was measured and found to have the temperature coefficient characteristic of ordinary thermal reactions over the range from 10°C. to 30°C.

3. The influence of the same temperatures upon the rate of ascorbic acid accumulation in turnip leaf discs in light of about 1400 foot-candles was also measured. At the lower temperatures, the temperature coefficient is greater than at higher temperatures.

4. Carbon dioxide is necessary for ascorbic acid accumulation in illuminated leaf discs.

5. The accumulation of ascorbic acid in turnip leaf discs requires light, and over the intensities used, the rate of accumulation increases as the light intensity is increased.

6. The results obtained with leaf discs from other plants also indicate that light influences the changes in ascorbic acid content.

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The Nature of the "Sporogenes Vitamin" and Other Factors in the Nutrition of *Clostridium sporogenes*[1]

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INTRODUCTION

In 1933 Knight and Fildes (1) discovered a factor necessary for the growth of *Clostridium sporogenes* and named it "sporogenes vitamin." In a later paper, Fildes (2) reported that the factor was also required by *Clostridium botulinum*. In 1935 Pappenheimer (3) prepared concentrates of the "sporogenes vitamin" from pregnant mares' urine. His preparations had the properties of an unsaturated hydroxy acid with a molecular weight of about 200. Although not crystalline, his concentrate was markedly active; as little as 0.04γ/ml. of his best preparation was sufficient to promote visible growth of the test organism.

In 1940 Peterson *et al.* (4) reported that at least one constituent of the "sporogenes vitamin" appeared to be biotin, as good growth of a strain of *Cl. sporogenes* was obtained on addition of this growth factor. Lampen and Peterson (5) investigated the situation further and reported that only one strain of *Cl. sporogenes*, A.T.C. No. 459, responded to biotin. Three other strains failed to grow with biotin or with biotin and *p*-aminobenzoic acid. (The latter compound is required by many clostridia.) The nature of the factor or factors comprising the "sporogenes vitamin" hence is still unknown, and a reinvestigation of the nutrition of *Cl. sporogenes* appeared to be desirable.

[2] Present address: Chas. Pfizer and Company, Brooklyn, New York.

Experimental

Cultures

Cl. sporogenes No. 533 of the National Collection of Type Cultures, London, England, obtained through the American Type Culture Collection, was used in all the work, except where otherwise indicated. This is the culture employed by Knight and Fildes (1) in their original investigation and is designated as *Cl. sporogenes* No. 10,000 by the American Type Culture Collection. In addition to this strain, 7 cultures obtained from Dr. Elizabeth McCoy, of the Department of Agricultural Bacteriology of this University, were used: Nos. 1, 4, 12, 44, 459, H-2, and *Cl. parasporogenes* The cultures were maintained as spores on soil containing 2% CaCO₃.

Assay

A large loop of the soil stock culture was transferred to 10 ml. of freshly steamed medium of the following composition: glucose 1%, Casitone[3] 1%, Norit-treated acid-hydrolyzed casein 0.5%, L-tryptophan 0.004%, L-cystine 0.005%, biotin 40 mγ/100 ml., nicotinic acid 60 γ/100 ml., p-aminobenzoic acid 10 γ/100 ml., salt solution B (see Table I) 0.5 ml./100 ml., K_2HPO_4 0.7%, and sodim thioglycolate 0.1%. After anaerobic incubation at 37°C. for 15–20 hours the cells were centrifuged and resuspended in 10 ml. of freshly steamed physiological saline solution. This suspension had a turbidity reading (per cent light transmission) of approximately 40 on an Evelyn colorimeter as compared with a reading of 100 for the uninoculated medium. Two drops of the suspension were used for each tube in the assay. The inoculum was maintained on the above medium by daily transfer for one week, at the end of which time a fresh transfer was made from the soil stock culture.

Oat jars were used to maintain anaerobiosis for some of the work reported here, but a partially evacuated pyrogallol jar similar to that described by Housewright and Koser (6), in which the air is replaced by CO_2, was used for most of the experiments. Incubation was usually for 15–20 hours. In experiments in which the incubation was continued for several days, it was found that autolysis markedly decreased the turbidity. The tubes were removed from the anaerobic jar and shaken thoroughly. The turbidity was estimated in an Evelyn colorimeter containing a 660 mμ filter with the uninoculated basal medium set at 100. The readings were most accurate if a series of not more than 5 tubes was shaken just prior to reading.

Results

In Tables I and II the composition of various media used in this investigation is given. The pH of these media was adjusted to 6.5 before autoclaving. Slightly better growth was obtained if the initial pH was 7.0, but in media with 0.7% phosphate buffer the darker color which developed on autoclaving at this pH was undesirable for turbidimetric estimation.

[3] A product of Difco Laboratories, Inc., Detroit, Michigan.

TABLE I

Composition of Basal Media

Component[a]	Medium			
	H	M	M-C	P
Glucose, %	1.0	1.0	1.0	1.0
Salts A[b], ml./100 ml.	0.5	0.5	0.5	—
Salts B[b], ml./100 ml.	0.5	0.5	0.5	0.5
Asparagine, %	0.01	—	—	—
Adenine, %	0.001	—	—	—
Guanine, %	0.001	—	—	—
Uracil, %	0.001	—	—	—
Xanthine, %	0.001	—	—	—
Riboflavin, γ/100 ml.	20	—	—	—
Thiamine, γ/100 ml.	20	—	—	—
Nicotinic acid, γ/100 ml.	60	—	—	60
Pyridoxine, γ/100 ml.	120	—	—	—
Ca pantothenate, γ/100 ml.	40	—	—	—
Biotin, γ/100 ml.	0.04	—	0.04	0.04
p-Aminobenzoic acid, γ/100 ml.	1.0	—	—	10
Pteroylglutamic acid, γ/100 ml.	0.1	—	—	—
Na thioglycolate, %	0.1	0.1	0.1	0.1
Tryptophan, %	—	—	0.004	0.004
Cystine, %	—	—	0.005	0.005
Amino acids	A	B	—	—
Hydrolyzed casein[c], %	—	—	0.5	0.5
K_2HPO_4, %	—	—	—	0.7

[a] The amino acids, vitamins, *etc.*, used were the biologically-active, or "natural" isomers except as noted in Table II.

[b] Salts A contains 25 g. K_2HPO_4 and 25 g. KH_2PO_4 dissolved in 250 ml. of water. Salts B contains 1.0 g. $MgSO_4\cdot7H_2O$, 0.5 g. NaCl, 0.5 g. $FeSO_4\cdot7H_2O$, and 0.5 g. $MnSO_4\cdot4H_2O$ dissolved in 250 ml. of water.

[c] The acid-hydrolyzed casein was prepared as follows: 100 g. of Labco Vitamin-Free Casein, 125 ml. of concentrated H_2SO_4, and 375 ml. of water were autoclaved at 120°C. for 15 hours. The H_2SO_4 was removed with $Ba(OH)_2$ and the filter cake thoroughly washed with hot water. The filtrate and washings were combined and treated at pH 3.0 with 1.0 g./100 ml. of Norit A for 1 hour.

Biotin Requirement

Initially, Medium H, a chemically defined medium containing most of the known microbiological vitamins and stimulatory factors, was used. The omission of single vitamins from this medium indicated that

TABLE II

Amino Acid Mixtures

Amino acid	A Per cent	B Per cent
L-Tryptophan	0.018[a]	0.004
L-Cystine	0.002	0.006
Glycine	0.003	0.020
DL-Alanine	0.056	0.012
DL-Leucine	0.120	0.017
DL-Isoleucine	0.065	0.009
DL-Serine	0.065	0.014
DL-Threonine	0.040	
DL-Phenylalanine	0.052	0.008
DL-Lysine HCl	0.068	0.009[b]
DL-Methionine	0.035	0.007
DL-Glutamic acid	0.228	
DL-Valine	0.070	0.015
DL-Aspartic acid	0.063	0.018
L-Arginine HCl	0.020	0.005
L-Histidine HCl	0.012	0.005
L-Tyrosine	0.032	0.005
L-Proline	0.041	0.015
L-Hydroxyproline	0.010	

[a] DL mixture used.

[b] L-isomer used.

Note: Mixture A contains amino acids in concentrations equivalent to 0.5% hydrolyzed casein in the medium. Where the DL-form was used, twice the quantity occurring in casein was used. Mixture B contains amino acids in the concentrations recommended by Fildes and Richardson (13) with isoleucine added.

biotin was the essential vitamin. Very little growth was obtained on this medium or on similar media containing hydrolyzed gelatin or casein in place of amino acids when biotin was omitted.

Eight strains of *Cl. sporogenes* were tested on a chemically defined biotin medium (M), with and without additional vitamins. Three series were set up for each culture and 10% serial transfers were made daily to fresh tubes of media. The first series contained medium M, the second medium M plus the other vitamins used in medium H, and the third medium M plus all of the other B vitamins. Table III shows that, with biotin present, growth, though scant, could nevertheless be maintained during continuous transfer without biotin, no growth was apparent after 3 transfers.

Strain No. 459 gave much heavier growth on this medium than did the others. It was later found that this culture would grow on a medium

TABLE III

Necessity of Biotin for Various Strains of Cl. sporogenes

Medium	Transfer	Strain							
		A.T.C. 10,000	1	4	12	44	A.T.C. 459	H-2	Para
M	1	90	91	90	91	92	73	92	90
	2	94	95	92	93	96	72	93	95
	3	93	91	94	90	95	62	94	93
	4	93	91	90	93	94	56	93	91
	5	90	84	88	94	94	50	90	90
M + Vitamins	1	92	91	91	91	93	78	93	90
	2	90	94	94	93	94	51	94	93
	3	92	92	91	92	92	47	91	91
	4	91	90	91	91	92		90	90
	5	90	87	86	90	92	38	91	89
M + Vitamins, except biotin	1	99	97	96	95	96	98	100	96
	2	100	99	100	100	100	98	98	98
	3	100	100	100	100	100	100	100	100

consisting only of biotin, inorganic salts, glucose, sodium thioglycolate, and buffer. It appears to be unique in not requiring any amino acids, in the medium. This and other characteristics noted later raises a question as to the validity of its classification in the species, *Cl. sporogenes*.

Stimulatory Effect of Other Vitamins

Thiamine, riboflavin, pantothenic acid, nicotinic acid, pyridoxine, *p*-aminobenzoic acid (PABA), pteroylglutamic acid (PGA), inositol, adenine, guanine, uracil, and xanthine were tested for stimulatory effect on medium M-C. Of these nicotinic acid, PABA and inositol appeared to be stimulatory. The greatest effect was given by the first 2 compounds. Inositol appeared to be mildly stimulatory at times. In Table IV the quantities of nicotinic acid and PABA required to give maximum stimulation are shown. Since PGA includes a PABA residue in the molecule (7), synthetic PGA [4] was tested to ascertain if it could replace PABA; but it failed to do so.

[4] Obtained from Lederle Laboratories Division, American Cyanamide Company, Pearl River, N. Y.

TABLE IV

Response to Nicotinic Acid, PABA, and PGA

An excess of PABA was included in the medium when testing the response to nicotinic acid and an excess of nicotinic acid was used when testing the response to PABA and PGA.

PABA	G	Nicotinic acid	G	PGA	G
$m\gamma/10\ ml.$		$\gamma/10\ ml.$		$\gamma/10\ ml.$	
0.1	82	0.05	86	0.1	86
0.5	81	0.1	84	0.5	84
1.0	79	0.5	81	5.0	84
2.0	78	1.0	79	50.0	84
10.0	78	5.0	79		

	Basal medium M-C	89
	All vitamins[a] added	78

[a] The following vitamin concentrations/10 ml. were used here: PABA, 0.1 γ, nicotinic acid 6 γ, inositol 10 γ, thiamine 4 γ, PGA 0.01 γ, riboflavin 4γ, Ca pantothenate 4 γ, pyridoxine 12 γ.

Replacement of Biotin

Numerous papers have been published showing that biotin can be replaced by oxybiotin (8, 9) and oleic acid (10, 11), to cite only recent papers, in the nutrition of microorganisms. Since *Cl. sporogenes* A.T.C. No. 10,000 was the strain of particular interest in this investigation, the amount of biotin required for maximum growth and its replaceability by oxybiotin and oleic acid were studied with Medium P. From

TABLE V

Response of Cl. sporogenes to Biotin and Oxybiotin

d-Biotin (A)	dl-Oxybiotin (B)	Galvanometer readings for	
		A	B
$m\gamma/10\ ml.$	$m\gamma/10\ ml.$		
None	None	100	100
0.2	1.0	96	92
0.4	2.0	87	85
0.6	3.0	82	75
0.8	4.0	74	74
1.0	5.0	75	74

Table V it can be seen that *dl*-oxybiotin[5] replaced biotin when added in sufficient concentration. One mγ and 2 mγ levels of oxybiotin were equivalent to 0.24 mγ and 0.47 mγ of biotin, respectively, so that a potency of approximately 48% of that of biotin can be assigned to oxybiotin on the assumption that only one isomer is active.

Biotin was replaceable by oleic acid but the effect was not easily reproducible. The erratic responses could be eliminated by the use of Tween 40, a water-soluble fatty acid derivative of sorbitan (product of Atlas Powder Company). In a recent paper Williams *et al.* (11) have shown the marked improvement in the response of lactic acid bacteria to oleic acid when Tween is included in the medium. The results of a typical experiment are given in Table VI. Oleic acid gave moderate growth, corres-

TABLE VI

Response of Cl. sporogenes to Biotin, Tween 40, and Oleic Acid,
Separately or in Various Mixtures

Tween 40	Oleic acid	*d*-Biotin	Galvanometer reading
mg./10 ml.	*γ/10 ml.*	*mγ/10 ml.*	
0	0	0	97
0	0	0.5	89
0	0	2.5	74
0	0	12.5	75
0	0	62.5	73
5	0	0	99
5	20	0	89
5	40	0	84
5	60	0	72
5	80	0	71
1.00	80	0	72
0.500	80	0	76
0.250	80	0	77
0.125	80	0	77
5	40	0.5	86
5	40	2.5	82
5	40	12.5	85
5	40	62.5	87
5	0	0.5	99
5	0	2.5	95
5	0	12.5	96
5	0	62.5	94
0.500	0	0	100
0.500	0	125	100
0.500	0	1250	96
0.500	0	12,500	99

[5] Obtained from Hoffmann-LaRoche, Inc., Nutley, N. J.

ponding to a galvanometer reading of 71, which is about the same as that obtained with biotin. The question naturally arose as to whether the 2 growth-promoting compounds would supplement one another. The data show that they did not do so. With 40γ of oleic acid and large additions of biotin no more growth was obtained than with oleic acid alone. It is evident that either Tween or oleic acid inhibited the action of biotin. The fifth series in the table shows that Tween has this property. For example, 2.5 mγ of biotin alone gave a reading of 74, but with 5 mg. of Tween the reading was 95, which is practically that of the basal medium. Larger quantities of biotin likewise failed to overcome the inhibiting action of Tween. In the sixth series the amount of Tween was greatly reduced and the biotin enormously increased. Even with 5000 times the quantity of biotin needed for maximum growth, multiplication of cells was inhibited. At present, we do not have sufficient data to explain the action of Tween 40. Perhaps it acts in the manner suggested by Kodicek and Worden (12) for oleic acid, *i.e.*, the formation of a layer of Tween molecules surrounding the bacterial cell and preventing the entrance of biotin into the cell.

An unexpected but satisfying outcome of the results with Tween 40 is the elimination of the possibility that oleic acid owes its effect to a small biotin impurity. It is obvious that biotin could not be the active agent in the oleic acid stimulation, since biotin is inhibited by Tween 40.

Necessity of Isoleucine

Fildes and Richardson (13) concluded from their study on the amino acid requirements of *Cl. sporogenes* that tryptophan, leucine, phenylalanine, tyrosine, and arginine were indispensable and that histidine, cystine, methionine, and valine were important for good growth. Their amino acid mixture also included alanine, glycine, proline, serine, aspartic acid, and lysine. In our hands only very scant growth could be obtained with this amino acid mixture. However, since mixtures of amino acids in the proportion found in casein gave moderate growth, it was evident that some additional amino acid was required. Eventually, isoleucine was found to be the missing essential amino acid. Additions of either 5 or 10 mg. of isoleucine/10 ml. of medium M (this contained the Fildes-Richardson amino acid mixture) increased the growth from a reading of 96 to one of 87 on the Evelyn scale.

Unknown Stimulatory Factors

It was noted early in the work that Bacto fluid thioglycolate medium produced a much more luxuriant growth than was attained in the various synthetic or semisynthetic media. For example, the following turbidities as measured on an Evelyn colorimeter are representative:

Medium M 80–85, Medium P 70–75, Bacto fluid thioglycolate medium 30–35. Investigation of the components of the Bacto medium disclosed that Casitone (a pancreatic digest of casein) was responsible for the enchanced growth. Several tryptic digests of casein were then prepared and found to possess comparable activity.

Since Casitone is marketed as a dry powder and possesses a fairly uniform stimulatory activity, it was chosen as a standard material upon which estimates of stimulatory activity of various preparations could be based. In studying the distribution and release of the unknown factor, Medium P was used as the basal medium and a standard
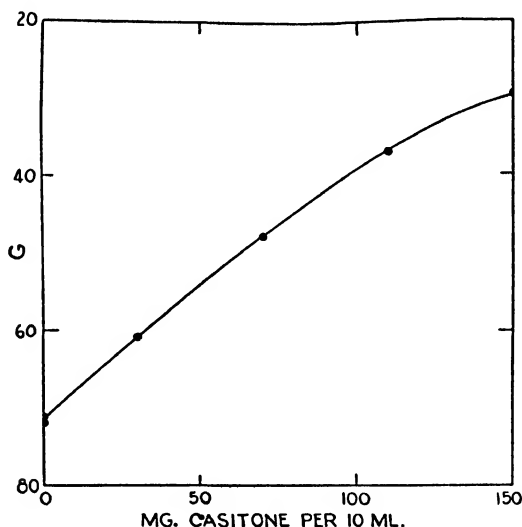
FIG. 1. Response of *Cl. sporogenes* to Casitone.

curve obtained with graded amounts of Casitone was included in each assay. A unit (U) of activity was arbitrarily set as the response of *Cl. sporogenes* A.T.C. No. 10,000 to 1 mg. of Casitone in 10 ml. of medium P. A typical Casitone curve, given in Fig. 1 shows that high concentrations were required to produce maximum growth.

The stimulatory factor was found to occur in a variety of partial protein digests as well as in certain other products. Of several materials tested, only corn steep liquor appeared to be superior to Casitone on a dry weight basis. Digestion of several proteins with trypsin gave the following potencies expressed in units/mg. based on the original weight

of protein used: Lilly insulin 1.34, lactalbumin 0.62, gliadin 0.53, Labco casein 0.48, Merck egg albumin 0.24, and Difco gelatin 0.19. In Table VII the activity of acid hydrolyzates of various proteins is given.

TABLE VII

Activity of Acid Hydrolyzates of Proteins

1 N HCl at 100°C. for 24 hours with an acid:protein ratio of 10:1 was used in all cases. The digests were neutralized with NaOH and filtered. The total nitrogen (Kjeldahl) content of each filtrate was determined.

Protein	U/mg. original protein	U/mg. sol. N
Lilly insulin	0.7[a]	27[a]
Labco casein	2.3	22
Difco isoelectric casein	2.2	21.5
Difco gelatin	1.8	13
Merck egg albumin	2.2	27
Fibrin[b]	1.8	21.5
Lactalbumin	1.5	20
Gliadin	1.5	19
Lilly salmine protamine	1.0	7

[a] This digest behaved peculiarly. The potency figures in U/ml. on successive assays were: 98, 75, 24. The figures given in the table represent an average of the three assays. No such change in potency was noted in the other hydrolyzates.

[b] A sample of purified fibrin obtained from Dr. D. V. Frost, Abbott Laboratories, North Chicago, Illinois.

To compare the ability of trypsin, pepsin and papain to release the stimulatory factor from casein, experiments were performed in which the enzymes were incubated with casein and aliquots removed at intervals and assayed. In Table VIII results of a typical experiment are given. The data indicate that papain liberated the stimulatory factor most effectively.

The occurrence of the unidentified factor in a variety of partial protein hydrolyzates suggested a molecule embodying a peptide structure. To determine whether the molecule could be further degraded enzymatically as, for example, by peptidases, hog intestinal mucosa[6] and partial hydrolyzates were incubated together. The activity was found to decrease to about 50% of its initial value while the α-amino nitrogen values increased 3-fold. Even with prolonged

[6] The intestinal mucosa, obtained from Oscar Mayer and Company, Madison, Wis., was immediately frozen and maintained in this condition until used.

TABLE VIII

Enzymatic Release of Factor from Casein

5 g. of Labco casein were suspended in 50 ml. of water containing buffer and activator (where used) and incubated with shaking at 37°C. with the enzyme.

Enzyme	Buffer	pH	Activator
50 mg. trypsin	1% NaHCO$_3$	8.5	None
50 mg. pepsin	None (HCl)	2.0	None
325 mg. papain	1% Na acetate	·5.0	50 mg. Na thioglycolate

Incubation	Trypsin	Pepsin	Papain
Hours	*U/ml.*	*U/ml.*	*U/ml.*
3	87	83	92
6	78		196
9.5	66	130	245
12.5	88	142	260
24	84	160	260
55	134	182	262
72	130	176	300
Enzyme blank	1	3	12

incubation periods and large quantities of the mucosa, roughly 50% of the activity remained.

Mild acid hydrolysis also released the factor. A comparison of HCl and H$_2$SO$_4$ for this purpose is given in Table IX. Hydrochloric acid released the factor more rapidly than sulfuric, as would be expected from its superior catalysis of protein hydrolysis. Maximum potency was reached at about the same extent of hydrolysis, irrespective of which acid was used. Assuming that about 70% of the soluble nitrogen should be α-NH$_2$-N if hydrolysis were complete, it appears that about 90% of the peptide linkages were hydrolyzed when maximum potency was reached. This suggests that the growth-promoting factor, if a peptide, is a short one. Of course there are many other possibilities and these data should be regarded as merely suggestive.

Various other conditions of hydrolysis, such as higher temperature or concentration of acid, were tried but, in general, gave hydrolyzates of lower potency than those listed in Table IX. Barium hydroxide was also tried as a hydrolytic agent but the potencies obtained were so low that they are not included in the table.

Attempts at concentration of the factor from enzymatic or mild acid digests of casein were unsuccessful. The activity per mg. of nitrogen never increased over 2- or 3-fold. The activity seemed to be distributed in several fractions and only occasionally was a clear-cut separation obtained. The fractionation procedures tried involved butanol and phenol extraction, Neuberg precipitation, precipitation with acetone,

TABLE IX

Release of Factor by HCl and H$_2$SO$_4$

50 g. of Labco casein were suspended in 500 ml. of 1 N acid and maintained at 100°C. Samples of the hydrolyzates were neutralized with KOH. The figures are for the undiluted aliquots.

Digestion	HCl			H$_2$SO$_4$		
	U/ml.	Sol. N.	α-NH$_2$-N^a	U/ml.	Sol. N.	α-NH$_2$-N
Hours		*mg./ml.*	*mg./ml.*		*mg./ml.*	*mg./ml.*
2	100	10.80		28	4.48	1.62
4	180	11.40	3.66	130	10.62	2.70
6	206	10.20	3.46	136	11.96	2.70
10	254	11.40	4.32	258	11.40	3.66
20.5	364		5.56	284	9.90	4.68
24	344	10.36	6.84	290	11.80	
48	252	11.80	7.42	350	11.80	6.04
72	218	10.80	8.32	272	10.26	6.48

^{a} NH$_3$ was not removed prior to determination of α-NH$_2$-N.

barium-methanol, metal salts, and phosphotungstic acid, adsorption (single step and chromatographic), dialysis, and Craig countercurrent distribution.

Relation of Stimulatory Factor to Strepogenin

The resemblance between the stimulatory factor and strepogenin (14, 15, 16) was early apparent. A strepogenin concentrate prepared from casein hydrolyzate by the method of Sprince and Woolley (15) also exhibited activity for *Cl. sporogenes*. A number of protein digests as well as specially treated fractions of protein digests were assayed by both *Cl. sporogenes* and *L. casei* to determine whether the comparative potencies of the different preparations were the same. Table X summarizes some of these data. The differences between the two assays are more numerous and marked than the agreements. If the two factors were identical, the activity ratios for any two preparations should be about the same for both test organisms. For example, insulin was over 2.5 times as active as lactalbumin for *L. casei* while for *Cl. sporogenes* the two preparations possessed comparable activity. Preparations 25-F and 31-B were high in "sporogenes vitamin" as compared with their content of strepogenin. A factor which might account for the difference in response of the two organisms is the fact that *Cl.*

sporogenes is actively proteolytic while *L. casei* is not. Thus, the former could quite possibly utilize long chain peptides containing the active nucleus by enzymatically splitting off the extra amino acids. However, it seems more probable that the two factors are different since there are so many discrepancies in the two assays.

TABLE X

Activity of Various Preparations for Cl. sporogenes and L. casei

Preparation[a]	Cl. sporogenes		L. casei[b]	
	No. of assays	U/ml.	No. of assays	U/ml.
Insulin digest	2	3.8	2	11.8
Casein digest	2	1.8	2	3.4
Gelatin digest	2	0.8	1	0.7
Lactalbumin digest	2	4.0	2	4.4
No. 12-D	2	71	1	2.1
No. 18-A	3	30	2	6.1
No. 20-D	3	13	2	6.9
No.21-A	3	29	2	5.4
No. 22-A	3	29	1	0.9
No. 24	3	21	2	1.6
No. 25-F	4	237	2	3.8
No. 27	4	346	1	7.5
No. 30-A	2	13	1	3.2
No. 31-A	3	121	1	4.5
No. 31-B	3	191	1	3.4
No. 31-C	3	201	1	6.4

[a] The numbered preparations represent various fractions from isolation work.

[b] For convenience 1 unit (U) is defined as equivalent to the growth obtained when 1 mg./10 ml. of Wilson's Liver L was added to the basal medium. The assay method was that described by Sprince and Woolley (14).

Additional Stimulatory Agents Tested

In addition to the vitamins and stimulatory factors mentioned earlier, the following compounds were also tested: nucleic acid, adenosine, adenylic acid, guanosine, guanylic acid, glutamine, glutathione, and inorganic ammonium salts such as $(NH_4)_2SO_4$ and NH_4Cl. All of these compounds, except guanosine and guanylic acid, provided a mild stimulatory action. Two anti-pernicious anemia preparations were assayed in the hope that a correlation might exist between the activity

of these preparations and the response of the test organism. One unit of Lilly Reticulogen assayed 14 units of the *Cl. sporogenes* stimulatory factor, and 1 unit of Sharpe and Dohme Anti-Pernicious Anemia factor assayed 22 units. These potencies are quite low when compared with the anti-pernicious anemia activity of the preparations.

DISCUSSION

From the data presented in this paper, it can be seen that the nature of the "sporogenes vitamin" depends largely on the index of growth that is used. If scant growth (G = 85–95) is taken as the criterion, then either biotin or oleic acid satisfies the requirements. Either of these can function as the sole indispensable vitamin. If moderate growth (G = 70–80) is used as the standard, then PABA and nicotinic acid are required to supplement the biotin or oleic acid. For heavy growth (G = 30 or lower), the unidentified factor found in partial protein digests is also required.

Pappenheimer's (3) results are best explained on the basis that his concentrate contained either biotin or fatty acid or both. He did not obtain any tests for nitrogen or sulfur, both of which are contained in biotin ($C_{10}H_{16}O_3N_2S$), but, if the concentrate contained only a fraction of a per cent of biotin, the quantity of nitrogen or of sulfur present in the test sample might have been too little to be detectable.

In addition to biotin or certain unsaturated fatty acids, certain amino acids are required by most strains of *Cl. sporogenes*. No effort has been made to determine which amino acids are necessary and which are stimulatory since Fildes and Richardson (13) have already investigated this phase of the nutrition of the organism. The beneficial effect of isoleucine as a supplement to the Fildes-Richardson amino acid mixture was the only new observation along this line.

SUMMARY

Moderate growth of eight strains of *Clostridium sporogenes* has been obtained on a chemically defined medium containing amino acids, glucose, biotin, PABA, nicotinic acid, salts, buffer, and sodium thiogly-

colate. Biotin is the only vitamin for which an absolute requirement exists. PABA and nicotinic acid exert a mild stimulatory effect on the growth of the organism. For heavy growth an unidentified factor found in partial protein digests was necessary. The occurrence and release of this factor are described.

Oxybiotin in higher concentrations effectively replaces biotin for the organism.

Oleic acid can also replace biotin in the medium. The possible relationship of biotin, oleic acid and the concentrates of "sporogenes vitamin" prepared by Pappenheimer is discussed.

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Studies on the Respiration of the White Potato.[1] I. Preliminary Investigation of the Endogenous Respiration of Potato Slices and Catechol Oxidase Activity

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INTRODUCTION

Ever since Boswell and Whiting (1), in 1938, published their initial work on the terminal oxidase system in the tubers of white potato (*Solanum tuberosum* L.), the view that catechol oxidase (tyrosinase (2, 3)) plays a significant, if not predominant, part in the endogenous respiration of this vegetable has been widely accepted (4, 5, 6, 7, 8, 9). While Baker and Nelson (5) found fault with the evidence on which Boswell and Whiting (1) based their conclusions, they nevertheless agree that catechol oxidase is importantly implicated in the respiration of the potato (*cf.* 10). The emphasis of the later work has been on what substituted catechols might indeed be "acting as redox bodies in association with catechol oxidase in the respiratory system of potatoes" (8).

A study of the enzymatic systems involved in the "greying" of white potato dice during their dehydration (11) presented us with an opportunity to investigate for ourselves the response of potato slices suspended in phosphate buffer to catechol addition. Our observations have led us to question seriously the validity of the experimental basis on which Boswell and Whiting (1) promulgated the now generally accepted view of the qualitative and quantitative participation of catechol oxidase in potato respiration.

METHODS

Throughout the course of the work reported here, a number of different potato varieties was used, although the Maine Green Mountain variety served for the

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majority of the data. Within the limits of this investigation, neither the variety of the potato nor the season of the year in which the potatoes were obtained from the market appeared to affect the results.

Potato slices with an average diameter of 7.7 mm. and 0.5 mm. thick were razored on a hand microtome from cylinders punched out of tubers with a No. 4 cork borer. The slices were then suspended and washed in fast running tap water at 10–15°C. for different periods of time.

A Warburg apparatus was used to follow the respiration of the potato slices suspended in 0.01 M potassium phosphate buffer, pH 6.1 at 31°C. Twenty-five slices in 2.5–3.0 cc. of buffer were used per vessel; solutions of appropriate substrates were added from side arms.

To determine directly the amount of catechol destroyed through oxidation by catecholase, the method of Volterra (12) for the determination of phenols was employed. To 10 cc. of the test sample in a Klett colorimeter tube were added 0.5 cc. of the Folin and Ciocalteu phenol reagent,[2] (diluted 1:14 with distilled water) and 2 cc. of a 2% Na_2CO_3 solution. The tube was then shaken and placed in a boiling water bath for exactly one minute after which time the tube was cooled immediately in an ice bath for 3 minutes. The blue color developed was then read in a Klett-Summerson colorimeter against a reagent blank with a 660 mμ filter. The colorimeter reading was converted into γ of catechol from a standard curve.

RESULTS

A. Endogenous Respiration as Affected by the Washing Time

Boswell and Whiting (1), to obtain reproducible results with potato slices, found it desirable to wash them with running tap water for a period of time in excess of 4 hours. Similarly, Baker and Nelson (5) adopted a 20 hour wash as standard procedure. Both groups of workers report that the endogenous rates of respiration of their washed slices in phosphate buffer were maintained practically constant for periods of time up to 6 hours.

In our experience, potato slices, when washed for 15–96 hours in running tap water at 10–15°C., showed an initial endogenous respiration in phosphate buffer at 31°C. 2–2.5 times that shown by slices washed only 1–5 hours in running tap water. However, throughout at least 26 experiments where observations were made of respiration rates of slices in phosphate buffer for periods of time extending from 1 to 8 hours, the endogenous rate of respiration, whether initially low or high, irrespective of variety of potato employed or the season of the year, increased with time. In general, the percentage increase with time was greatest when the initial rate was low, whence it appeared that a

[2] Eimer and Amend, Inc., New York.

maximum endogenous rate was being approached in all cases and was most nearly realized with those slices which had been washed for 15 or more hours in running tap water and then observed in the Warburg vessels for 7 or 8 hours in phosphate buffer. Curves of typical results obtained with slices washed for 1, 17 and 24 hours are given in Fig. 1.
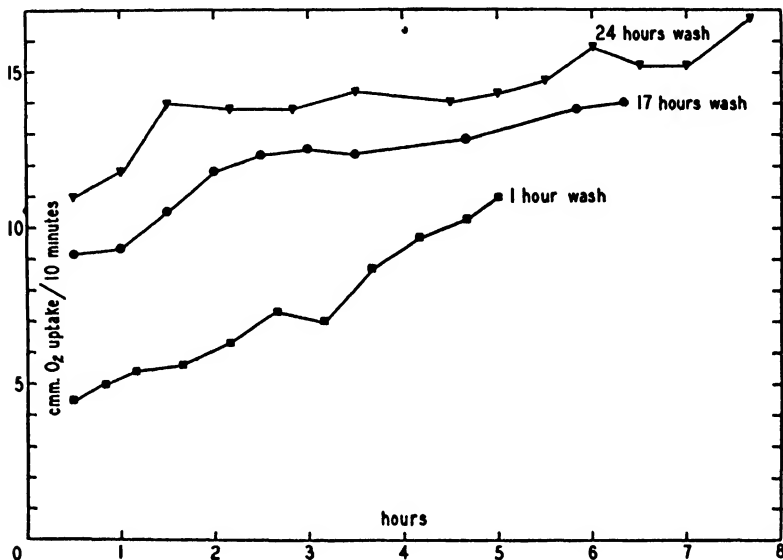

Fig. 1. Endogenous respiration of potato slices as affected by the washing time and length of time of observation under the given experimental conditions. 25 potato slices, washed in running tap water, 10–15°C., for the lengths of time indicated, were suspended in 3.0 ml. of phosphate buffer (0.01 M, pH 6.1) at 31°C.

Comparison of these curves indicates that for the first 2 hours in phosphate buffer the increase in rate of respiration of slices washed for the three periods of time is similar (25–27%). The increase in rate of the 1 hour-washed slices is maintained beyond 3 hours while it falls off for the 17 and 24 hour-washed slices as they approach an apparent maximum. In later experiments, slices washed for 66 hours at 10–15°C. also showed a 26% rise in respiration rate over the first two hours at 31°C., while the respiration of 114 hour-washed slices rose 10%.

Consideration of these results shows that washing in tap water for 15 or more hours at 10–15°C. does increase the endogenous respiration of potato slices, but whether, as Boswell and Whiting maintain, this

increase is due to removal of contents of cut cells and "wound healing" or is referable to adaptations to changes of the environmental relations of the cells of the slices, is not known. In any case, the manner of the increase in rate of endogenous respiration of washed slices when put in phosphate buffer at 31°C. indicates a response of the living tissue to the rise in temperature until a maximum rate is approached. The observed maximum endogenous respiration is of prime importance for the interpretation of the rise in the rate of respiration upon addition of catechol to the medium.

B. *Respiratory Response of Potato Slices to Catechol Addition*

As has been previously observed by others, addition of catechol to washed slices in phosphate buffer causes an immediate increase in O_2 uptake followed by a rapid falling off of this uptake until a rate of oxygen consumption is reached which is considerably below that of the
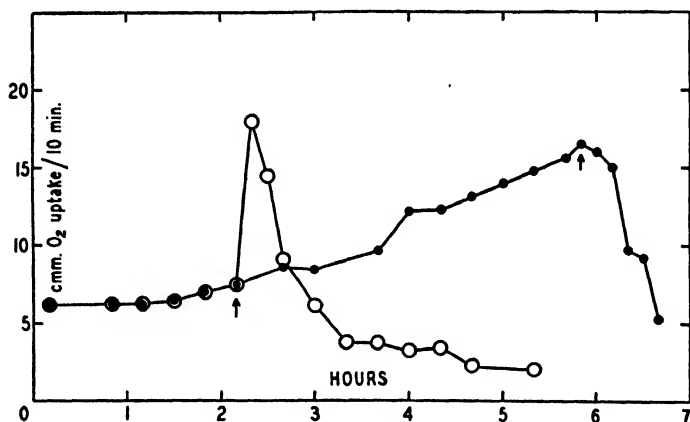
FIG. 2. Respiratory responses of potato slices to catechol addition before and at the time that the endogenous respiration.had approached the maximum rate attained as the result of the earlier catechol addition. The arrows indicate the times of addition of 2.2 mg. of catechol to 25 slices respiring in 3.0 ml. of phosphate buffer (0.01 *M*, pH 6.0) at 31°C.

initial endogenous rate. Fig. 2 illustrates data obtained when catechol was added to potato slices after approximately 2 hours in buffer. Observation for 6 hours of the endogenous respiration of the control slices revealed a rise to about the same level obtained 4 hours earlier

through catechol addition to similar slices. Of additional interest is the fact that, when catechol was added to these control slices, slight, if any, additional rise in oxygen consumption was noted, although the subsequent inhibition of respiration was apparently as rapid and effective as that exhibited by slices exposed to catechol after the first 2 hours.

Having observed failure of slices to respond to catechol addition at the time when their endogenous respiration had approximately reached its apparent maximum level, we determined what effect catechol would have on the rate of respiration if added to slices in phosphate buffer at some time before they had reached the apparent maximum rate. Fig. 3
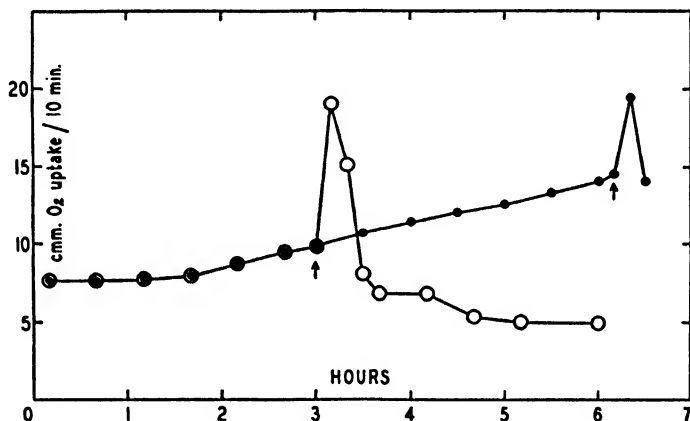
FIG. 3. Respiratory responses of potato slices to catechol addition early and later in the period of increase in the rate of their endogenous respiration. The arrows indicate the time of addition of 2.2 mg. of catechol to 25 slices respiring in 3.0 ml. of phosphate buffer (0.01 M, pH 6.0) at 31°C.

illustrates such a case. Whether catechol was added to slices whose endogenous respiration after 170 minutes in phosphate buffer was *ca.* 10 mm.[3] O_2/10 min. or to slices whose endogenous respiration after 390 minutes in phosphate buffer was 14.5 mm.[3] O_2/10 min., the maximum rate observed was the same, namely, 18.5 mm.[3] O_2/10 min. Hence, the amount of response to catechol addition in terms of increase of O_2 uptake appears to be conditioned by the extent to which the endogenous respiration rate has approached its possible maximum under the conditions of the experiment.

As Baker and Nelson (5) have shown, additions of different concentrations of catechol to slices respiring in phosphate buffer occasion

different increases in the rates of oxygen consumption—the greater the concentration, the larger the increase. Further, as the concentration of catechol initially added is less, the response of the slices to a second addition of catechol is greater. To illustrate this point a typical set of curves is given in Fig. 4. An addition of 2.2 mg. to the slices gave
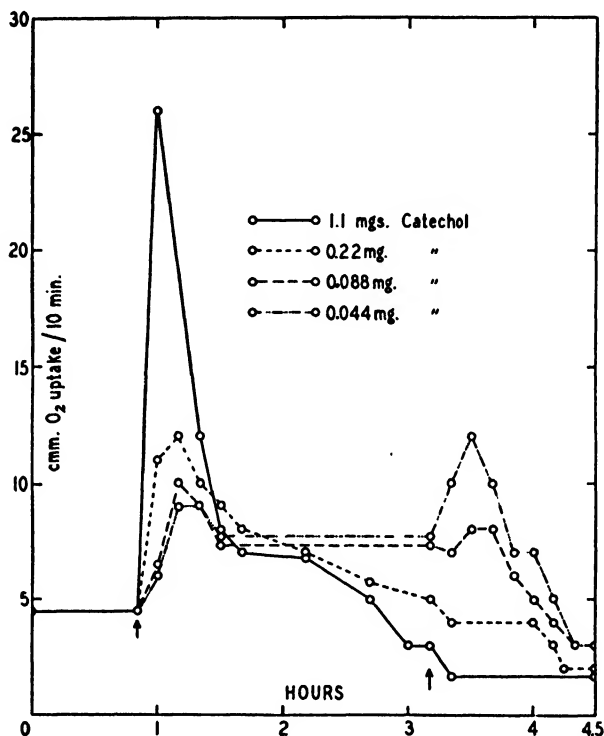

FIG. 4. Respiratory responses of potato slices to different amounts of catechol and relation of these responses to the effect of a subsequent addition of catechol. The first arrow shows the time of addition of the indicated amounts of catechol while the second arrow shows the time of addition of 2.2 mg. of catechol to the 25 slices respiring in 3.0 ml. of phosphate buffer (0.01 M pH 6.0) at 31°C.

results similar to those obtained with 1.1 mg. as shown. However, contrary to the mentioned authors' experience, the rate to which the oxygen uptake fell after the first addition of catechol was not the same irrespective of the amount added. Rather, the final rate prior to the second catechol addition of 2.2 mg. was higher as the initial catechol concentration had been smaller.

These results show that any attempt to determine what "per cent" of the total endogenous oxygen consumption is attributable to catecholase activity must, at least, take into consideration not only the maximum endogenous respiration rate of the tissue under the conditions of the experiment, but also the concentration of the catechol used to obtain the "catecholase response."

C. Oxidation of Catechol by Potato Slices

We have calculated that when 1.1 mg. of catechol are added to 25 slices (Fig. 4), the absolute quantity of oxygen consumed (51 ml.[3]) is equivalent to 200 γ of catechol oxidized for conversion to the final product (13). If, under these conditions, we assume that the available tissue is actually capable of oxidizing, as a non-toxic substrate, as much as 0.2 mg. of catechol, then we might expect to find that 0.02 mg. of catechol would be almost completely oxidized by the same amount of tissue.

TABLE I

Catechol Utilization by Potato Slices as Determined by the Phenol Test

Length of exposure min.	Initial catechol γ	Final catechol γ	Catechol oxidized [a] γ
70	0.0	4.7	—
70	19.1	21.2	2.6
70	950	700	254.7
120	0.0	3.3	—
120	19.0	16.4	5.9
120	950	798	155

[a] The amount of catechol oxidized is calculated by subtraction from the initial amount of catechol added the sum of the amount found in the control supernatant plus that found in the test solution.

Appropriate solutions of catechol in a total volume of 3.5 cc. were exposed to twenty-five 18 hour-washed slices of potato in Warburg flasks at 31°C. for both 70 and 120 minutes. We then assayed the supernatants for catechol. Table I presents the data obtained in duplicate experiments. From these results it is clear that although 25 slices were capable of destroying from 150 to 245 γ of catechol when exposed to 1000 γ of this phenol, the same number of slices did not oxidize more than 3–6 γ when exposed to 20 γ of catechol.

In view of the small amount of catechol apparently oxidized by the potato slices when exposed to 20 γ of the phenol under the above conditions, it might be concluded that such a small concentration would be without effect on the endogenous respiration of the slices. This is not the case. Fig. 5 shows that, while 20 γ of catechol do not occasion the

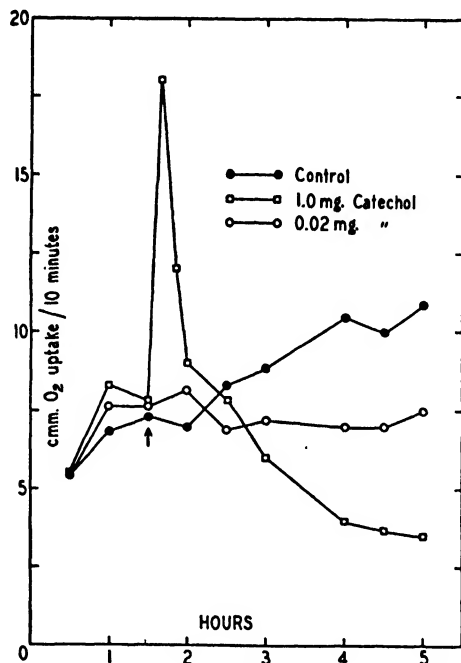Fig. 5. Effect of 1.0 and 0.02 mg. of catechol on the endogenous respiration of potato slices. The arrow shows the time of addition of the indicated amounts of catechol to the 25 slices respiring in 3.0 ml. of phosphate buffer (0.01 M, pH 6.0) at 31°C.

precipitous fall in the endogenous rate which results from the addition of 1000 γ to potato slices after the initial stimulation of oxygen uptake, nevertheless, the low catechol concentration does inhibit the normal increase of the endogenous respiration with time.

D. Effect of Catechol on the Catecholase Activity and Viability of Potato Tissue

It has been assumed that the fall in oxygen consumption by potato slices after treatment with catechol results from inhibition of the

catecholase by this substrate while the residual oxygen uptake is due to a system in which catechol oxidase plays no part (1). Nelson and Dawson (3) have pointed out that it is characteristic of purified catecholase to become inactivated or destroyed during the course of reaction with catechol. To determine whether the catechol oxidase activity of potato slices was irrevocably destroyed as a result of the addition of catechol in such a concentration as to evoke maximum response in oxygen uptake as well as in subsequent respiration decrease, we set up the following experiment.

One hundred potato slices were washed in running tap water for 1.5 hrs. At the end of the washing period, 50 slices were put in 20 cc. of 0.01 M phosphate buffer, pH 6.0, while the remaining 50 slices were put in a similar volume of buffer plus 4.4 mg. of catechol. Both flasks containing the slices were shaken for 1 hr. in a 31°C. water bath. At the end of this period of time, each set of slices was washed separately in running tap water for 2.5 hrs. Warburg vessels were then set up in duplicate pairs, one pair for the control slices and the other pair for the catechol-treated slices. Each vessel contained 25 potato slices, 3.0 cc. of phosphate buffer, pH 6.0, 2.2 mg. of catechol in 0.5 cc. in the side arm, and 0.3 cc. of 20% KOH in the inner well. After equilibration at 31°C., the endogenous respiration of the slices in the several flasks was recorded for three 10 min. periods, following which the catechol was added to the slices from the side arm. The oxygen uptakes for the 4 subsequent 10 min. periods were noted. The vessels were then removed from the bath and the slices carefully taken from each vessel and washed separately overnight in running tap water (16 hours). The slices were returned to Warburg vessels prepared in a manner similar to that given above. During the initial 50 min. period after equilibration in the Warburg bath at 31°C., 10 min. readings were made of the oxygen consumption and then 2.2 mg. of catechol were added to the slices in all 4 vessels. Subsequent oxygen uptakes were noted for seven 10 min. periods. Table II presents a summary of the data obtained.

TABLE II

Catechol Oxidase Activity of Potato Slices after Repeated Exposure to Catechol

	μl.O$_2$ consumption for 10 min. period	
History of slices	Before catechol addition	After catechol addition
1. Controls	9, 10, 11	27, 22, 11, 8
2. Once exposed	0, 0, 0	24, 22, 9, 6
3. Exposed once, washed overnight (Slices of (1))	0, 0, 0, 0, 0	26, 22, 9, 4, 4, 4, 3
4. Exposed twice, washed overnight (Slices of (2))	0, 0, 0, 0, 0	15, 8, 3, 3, 3, 1, 1

Consideration of these data leads to the conclusion that catechol, rather than effecting a destruction of catecholase and thus inhibiting

at least 66% of the observed respiration with the remainder to be accounted for by some other system (1), destroys the viability of the tissue itself without irreversibly inactivating the catechol oxidase. Once the potato cells have been exposed to such a concentration of catechol as effects the maximum response in oxygen uptake, the tissue, even after extended washing, no longer shows an endogenous respiration. On the other hand, the capacity of the apparently poisoned, washed slices to oxidize catechol may be little, if at all, impaired. Furthermore, it is evident that the oxygen consumption attributed to the so-called "residual" respiration of the catechol-treated slice may not, indeed, represent respiration, but be the result of enzymatic oxidation of the catechol by the nonviable slice tissue at a rate which decreases with time. It may be significant that the courses of oxygen uptake by the nonviable and viable tissues in response to catechol additions are markedly similar, despite the fact that their capacity for endogenous respiration is destroyed in one case and not in the other. The possibility is suggested that the observed reaction of the viable potato slice to catechol addition is merely an expression of its catechol oxidase capacity without necessary implication of this enzyme in the endogenous respiration of the slice.

DISCUSSION

It appears to us that the preceding observations can be understood if it is assumed that catechol is acting primarily as a cell poison, capable, in suitable concentration, of disrupting completely the normal metabolic processes on which the viability of the cells depends, and only secondarily as a substrate for tyrosinase. Contrary to Boswell and Whiting's (1) statement "that the tissue slices retain their turgidity under all concentrations of catechol and throughout the duration of the longer experiments" (3 hrs.), and the implication that viability of the tissue is maintained even when a concentration of 0.0036 M catechol is used, we have never failed to observe a decrease in the turgidity of the potato tissue after it had once been exposed to such a catechol concentration as is capable of eliciting the maximum respiration response. The extent of observable flaccidity of the tissue and the plasmolysis of its constituent cells increased with time and was directly related to the amount of respiration increase occasioned by the different levels of catechol employed. Conversely, the extent of

respiration increase apparently reflects the amount of potato tissue destroyed by the catechol. As less tissue is poisoned by smaller concentrations of catechol, the greater will be the respiratory response of such tissue to a succeeding catechol addition. Microscopic examinations of potato slices suspended in catechol solutions of increasing concentration revealed increasing amounts of brown discolored, apparently coagulated protoplasmic material, pronouncedly in the vascular tissue, but quite generally throughout all of the cells, especially with the highest catechol concentrations employed. The brownish colored depositions could not be removed by washing in running tap water for 24 hours.

Whether or not some oxidizing system capable of acting upon catechol as a substrate is involved in the endogenous respiration of potato, it is clear that because of concomitant injurious effects of this phenol on the viability of the tissue itself, catechol is an unsuitable substrate for the determination of this question. Further, in our opinion, there has been too general and uncritical an acceptance of the conclusions drawn from Boswell and Whiting's (1) work on catechol and endogenous respiration as to the identity and relative importance of the enzyme systems responsible for this respiration. . Results of investigations to characterize more satisfactorily the metabolic phenomena involved in potato respiration will be published subsequently from this laboratory.

ACKNOWLEDGMENT

SUMMARY

Catechol is an unsatisfactory substrate for the elucidation of the enzymatic systems involved in the endogenous respiration of the potato tuber. It inhibits reversibly the catecholase activity of potato tissue while destroying irreversibly the endogenous respiration. Hence, it appears that catechol acts primarily as a cell poison, capable of disrupting completely the normal metabolic processes on which the viability of the cells depends, and only secondarily as a substrate for tyrosinase. For us, the problem remains undecided as to what extent, if any, tyrosinase acts as the terminal oxidase in the endogenous respiration of potatoes.

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On the Action of Sulfanilamide. XII. A Set of Noncompetitive Sulfanilamide Antagonists for *Escherichia coli* [1]

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INTRODUCTION

In 1940, Woods (1) showed that a substance present in yeast-extract was able to counteract sulfanilamide (SA). This substance was supposed, and afterwards proven, to be *p*-aminobenzoic acid (PABA), which acted as a growth factor for *Clostridium acetobutylicum* (2) and several other bacteria (3). Woods and Fildes (4) supposed that the sulfonamides displace the "essential metabolite" PABA from the enzyme system in which it is involved. In this theory, PABA functions as a substrate. The competition between the inhibitor (SA) and the substrate (PABA) was proven by the constant ratio between the concentration of SA and the just neutralizing concentration of PABA (3).

Apart from PABA, several other substances showed SA antagonizing properties *e.g.*, peptone (5), methionine (6), purines (7), and various aminoacids (8).

Kohn and Harris (6) supposed that the biosynthesis of methionine was inhibited by SA, considering PABA as a catalyst rather than as a substrate.

The study of other drugs, inhibiting other enzyme reactions, led to the conception of competitive antagonists (competition between inhibitor-substrate or inhibitor-catalyst) and non-competitive antagonists (end products of the blocked reaction) (9).

Several *Lactobacilli* seemed to form a simple instance of this conception. According to Teply (10) the action of sulfapyridine on *L. arabinosus* was counteracted by a liver factor resembling folic acid. Lampen and Jones (11, 15) then showed with *Streptococcus faecalis*, *L. arabinosus* and several *Enterococci* that inhibition by sulfanilamide is antagonized completely and non-competitively by pteroylglutamic acid. Independently, we also found this true with *Streptobacterium plantarum*. It was concluded that the synthesis of pteroylglutamic acid from PABA is the major point of attack of sulfanilamide-inhibition with these organisms.

With other organisms the mechanism seemed more complicated. It was evident the number of actual facts grew, the matter became more confused. Shive (14, 17), in a induced mutant of *E. coli*, requiring PABA, the latter factor could be replaced by a

mixture of amino acids together with adenine, guanine, xanthine, hypoxanthine, and relatively high concentrations of thymine. Growth on this medium was highly resistant against sulfadiazine (12). Moreover, with *E. coli* pteroylglutamic acid is ineffective as an SA antagonist (11).

The inhibition of pteroylglutamic acid synthesis is not the only point of attack in *Lactobacilli*, as Snell demonstrated (13) that several purines (though inactive alone) were able to antagonize SA in the presence of suboptimal concentrations of PABA. As the number of actual facts grew, the matter became more confused. Shive (14, 17), in a general theory of drug inhibition, very concisely defined the problem by supposing that at increasing concentrations of inhibitor (I, related to the substrate S), an increasing number of enzyme reactions ($E_1 - E_n$) leading to the different end-products $P_1 P_2$ . . . P_n, is blocked. S acts as a competitive antagonist, and *ceteris paribus* the ratio I/S is constant over the whole range of concentrations.

In low concentrations of I the addition of P_1, the end-product of the most sensitive reaction (E_1), will restore growth. In higher concentrations of I, P_1 becomes inactive because E_2, etc., will also become inhibited. But as the complex between enzyme and inhibitor E_2I is more dissociated than E_1I (or less I is adsorbed on E_2 than on E_1) less S is necessary to restore growth when P_1 is present. So on addition of P_1 the ratio I/S will increase. P_2 will only be a non-competitive antagonist if P_1 is present and P_n will only show antagonistic action if P_1 to P_{n-1} are present. Shive than found with *E. coli* that methionine is the end-product of the first reaction inhibited by SA, as the ratio $\frac{(SA)}{PABA}$ was raised 3-fold by addition of methionine. A further 3-fold rise with xanthine or guanine showed that the second reaction blocked by SA led to a purine as its end-product. In higher concentrations, however, SA still inhibited growth, indicating that still other reactions were blocked.

This theory seemed to be able to explain many contradictory facts. For instance why methionine and xanthine apparently are SA-antagonists for most bacteria but not (or to a lesser extent, 13) for several *Lactobacilli* (15), and again why pteroylglutamic acid is a non-competitive SA antagonist for several *Lactobacilli*, but is ineffective with other bacteria. The *Lactobacilli* require complicated media for growth, including methionine and several purines. Evidently the anabolic reactions leading to these substances are lost in some of these organisms, leaving only the synthesis of pteroylglutamic acid to be inhibited by sulfanilamide. *Lactobacilli* which need pteroylglutamic acid are, of course, insensitive to sulfanilamide.

On the other hand, if, in *E. coli*, the synthesis of pteroylglutamic acid is inhibited but in rather high concentrations, the antagonistic action of this substance can only be found if all the previous antagonists (*i.e.*, the end-products of all the reactions inhibited by *lower* SA concentrations) are present.

It was the aim of our experiments with *E. coli* to fill in the gaps in the series between the known antagonists, methionine and xanthine, and the postulated antagonist, pteroylglutamic acid, in the hope of clarifying at least part of the confusing evidence on sulfanilamide antagonists.

EXPERIMENTAL

E. coli and *Salmonella typhimurium* were transferred weekly on broth-agar.

A standard droplet (0.03 ml.) of a 1:300 dilution of an 18–24 hour culture of *E. coli* or *Salmonella typhimurium* on synthetic medium, containing about 2×10^4 viable bacteria, was used as inoculum for 10 ml. of the final medium.

The basal medium contained: glucose, 20 g.; KH_2PO_4, 6 g.; K_2HPO_4, 6 g.; NH_4Cl, 2 g.; $MgSO_4 \cdot 7H_2O$, 50 mg.; $FeSO_4 \cdot HPO$, 5 mg.; and distilled water, 1000 ml.

The glucose was freed of PABA by Norit adsorption at pH 3.

The medium was sterilized by heating for 30 minutes at 115°C., except for the glucose, which was sterilized by filtration through an L5 glass filter and added sterilely after autoclaving.

Fifty ml. samples of the doubly concentrated medium were diluted with solutions of sulfanilamide and the various antagonists to a final volume of 100 ml.

Ten ml. of these mixtures were pipetted in glass tubes (diameter 2.7 cm., closed with metal caps). One standard droplet, containing the necessary amount of PABA, was added. After inoculation the mixtures were incubated at 37°C. Freshly prepared solutions of PABA sterilized by boiling for 3 minutes were used.

Synthetic pteroylglutamic acid[2] was used throughout these experiments. The pteroylglutamic acid solution was sterilized by filtration and kept in the, dark.

Growth was measured as turbidity in a Moll extinctometer. The correlation between cell concentration and galvanometer reading was linear up to a reading of 800.

RESULTS

With E. coli

The minimal concentration of SA which inhibits growth for 48 hours on the simple medium is 30 mg./l. As we specially wanted to study the enzyme systems which are inhibited by higher concentrations of sulfanilamide, 2000 mg./l. SA, was used in all experiments. (Still higher concentrations are unsuitable for our purpose as the obtained growth inhibition is no longer reversible by PABA).

As 3000 γ/l. PABA restored growth, the antibacterial index $\dfrac{(SA)}{(PABA)}$ was about 650 for *B. coli*. The shift of this index was studied by measuring growth in a series of tubes containing the supposed antagonists, 2000 mg./l. SA and varying concentrations of PABA. In accordance with Shive, we found that methionine raised the index about 10-fold. With methionine and xanthine a further increase (3- to 10-fold) was found. Xanthine alone is inactive. Methionine and xanthine thus probably are the end-products of the first and second enzyme systems

[2] The authors are indebted to the Lederle Laboratories Division, American Cyanamid Co., Pearl River, N. Y., for the synthetic pteroylglutamic acid.

inhibited by increasing SA concentrations. For the study of the enzyme-systems blocked by still higher SA concentrations (or under our conditions giving a further increase of the antibacterial index), it was necessary to determine the optimal concentrations of methionine and xanthine, giving the desired effect.

In Fig. 1 the growth (48 hours) of *E. coli* on the basal medium, containing 2000 mg./l. SA with suboptimal amounts of PABA (300, 100,
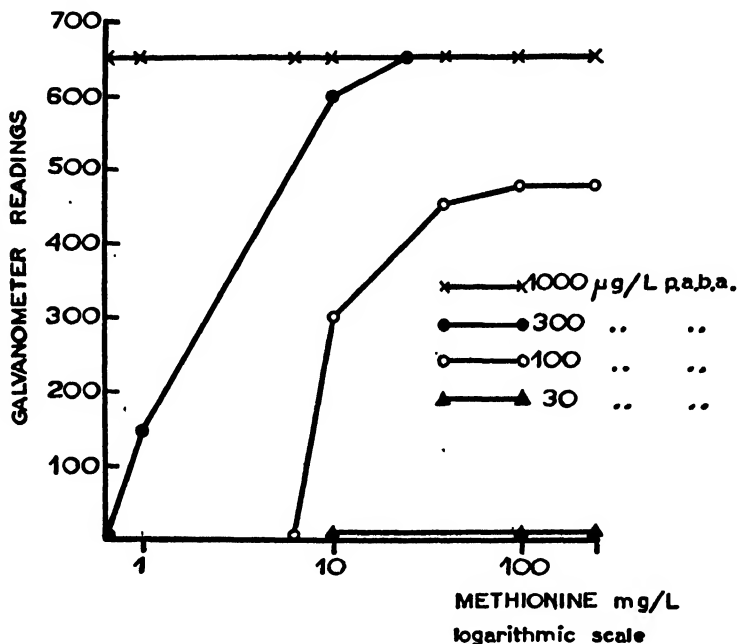

FIG. 1. *E. coli*. Basal medium + 2000 mg./l. SA. Estimation of the amount of methionine necessary to restore growth in the presence of suboptimal amounts of PABA.

and 30 γ/l.), is plotted against the concentration of methionine. A concentration of 1000 γ/l. of PABA will support growth without the addition of methionine. With 300 γ/l. of PABA, growth is a nearly linear function of the methionine concentration until with 35 mg./l. optimal growth is obtained. The antibacterial index has increased at least 3-fold. The remaining concentration of PABA is necessary to displace SA from the less sensitive enzyme systems. A concentration of 100 γ/l. PABA will

only partly unblock these other enzyme systems and, even in the presence of optimal methionine concentration, growth is not fully restored. With 10 γ of PABA the bacteria are unable to grow at all.

Using the basal medium with 35 mg./l. of methionine, the optimal concentration of xanthine could now be determined (Fig. 2). With a concentration of 25 mg./l. the index increased about 30-fold, only 3 γ/l. PABA being necessary for the unblocking of the remaining, less
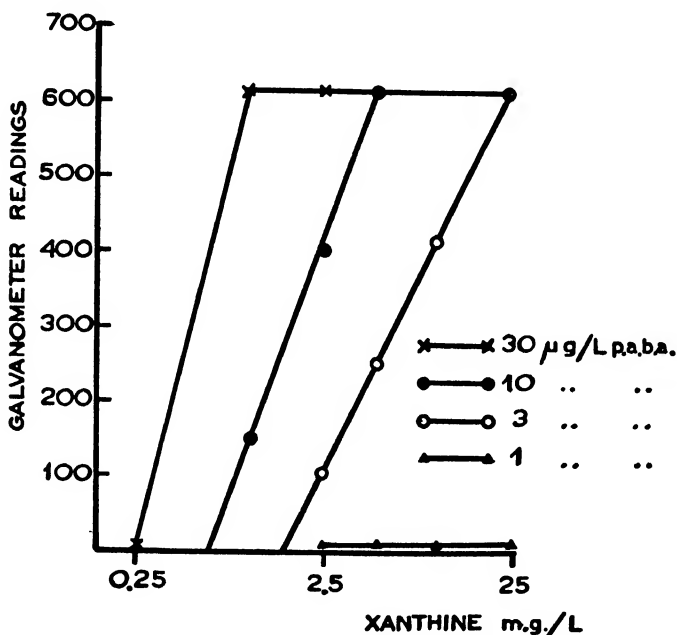

FIG. 2. *E. coli.* Basal medium + 2000 mg./l. SA. + 35 mg./l. methionine. Estimation of the amount of xanthine necessary to restore growth in the presence of the first antagonist methionine, and suboptimal amounts of PABA.

SA-sensitive, enzyme systems. With higher PABA concentrations the xanthine-producing system seems to be partly unblocked and full growth is obtained with lower xanthine concentrations.

We were able to ascertain that other purines, *e.g.*, adenine, hypoxanthine and guanine, were unable to antagonize SA to the same degree. Adenine and hypoxanthine were slightly toxic, guanine showed some effect. Thymine and uracil were without activity. The antagonizing action of mixtures of these compounds was not studied.

Looking for the third antagonist we used a medium containing opti-
mal concentrations of methionine and xanthine and tested 17 amino
acids, all known members of the vitamin B complex, choline, the
purines adenine, hypoxanthine and guanine, and the pyrimidines
uracil and thymine, for SA antagonistic activity. All compounds were
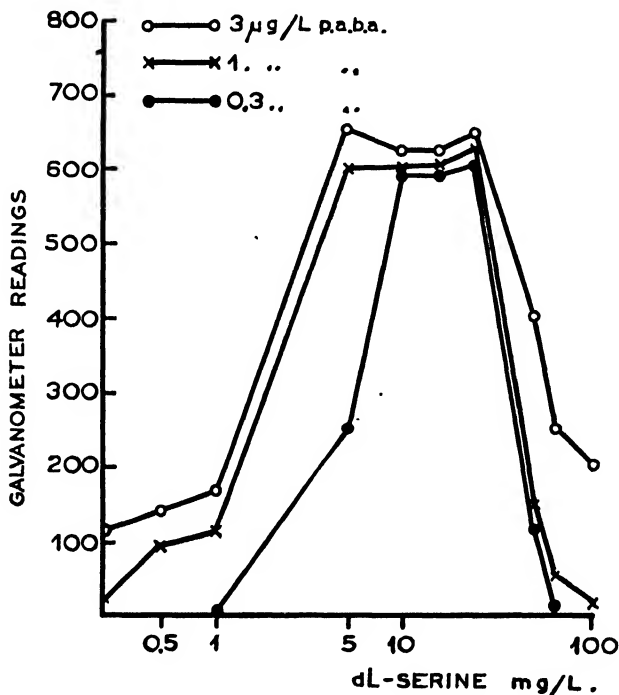tested in a concentration of 10 mg./l. except the "vitamins," which

Fig. 3. *E. coli.* Basal medium + 2000 mg./l. SA + 35 mg./l. methionine + 25
mg./l. xanthine. Eestimation of the amount of serine necessary to restore growth in
the presence of the first two antagonists and minute amounts of PABA.

were added in a concentration of 1 mg./l. Only serine showed a further
increase in the antibacterial index, indicating its SA antagonistic
activity. The optimal concentration of DL-serine is 15 mg./l. as is
shown in Fig. 3. On further increasing the concentration, serine
becomes toxic.

With methionine, xanthine and serine the antibacterial index has
increased to about 6×10^6; in this experiment only 0.3 γ/l. of PABA is

necessary for growth, that is, antagonizing SA for the remaining enzyme systems.

Proceeding as before, all compounds previously used were tested again in a medium containing the 3 antagonists already known. Two of the tested substances, *viz.*, thymine and pteroylglutamic acid, both showed SA antagonistic activity. With both substances optimal growth was obtained without PABA. The optimal concentration was determined as 30 mg./l. for thymine whereas 300 γ/l. of pteroylglutamic acid was necessary. In Fig. 4 a representative experiment is given,
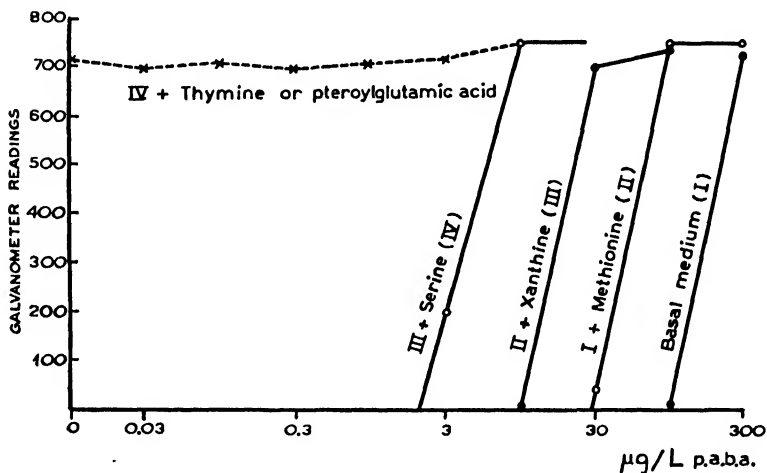
FIG. 4. *E. coli*. Basal medium with 2000 mg./l. SA. The amount of PABA necessary to restore growth decreases stepwise by the successive addition of methionine, xanthine, serine and pteroylglutamic acid *or* thymine until, in the presence of the 4 antagonists, growth is obtained in the absence of PABA.

showing the growth of *E. coli* as a function of the PABA cncentration in the presence of the 5 SA antagonists. At first glance it seems that with methionine, xanthine, serine and thymine, *or* pteroylglutamic acid the set of noncompetitive antagonists for *E. coli* is complete.

The postulated antagonism of pteroylglutamic acid is found indeed, but the necessary concentration of 300 γ/l. seems rather high. That the activity is not due to free PABA is indicated by the fact that after shorter incubation times pteroylglutamic acid is a very active SA antagonist in PABA concentrations which are much too high to be present in or derived from the pteroylglutamic acid. An experiment

with pteroylglutamic acid, to which various amounts of PABA had been purposely added, further substantiated our view.

With *Salmonella typhimurium* quite similar results were obtained.

DISCUSSION

The described experiments show that, for *E. coli*, a series of noncompetitive SA antagonists exists, comprising methionine, xanthine, serine and thymine, *or* pteroylglutamic acid in the order named, each substance showing antagonistic activity, only when the lower members of the series are present. In the presence of the former 3, however, thymine and pteroylglutamic acid are both active independently. Noncompetitive antagonism in the full sense (activity independent of the SA concentration) is, of course, only found for each substance if the 3 others are present.

One should realize that the finding of a noncompetitive antagonist as in the above case not necessarily means that the antagonist *is* the end-product of the blocked reaction. The bacterium might circumvent the blocked reaction by using the added substance as a substrate for another reaction, permitting growth.

Apart from this objection the following diagram illustrates the supposed relations. The four enzyme systems are inhibited by increas-
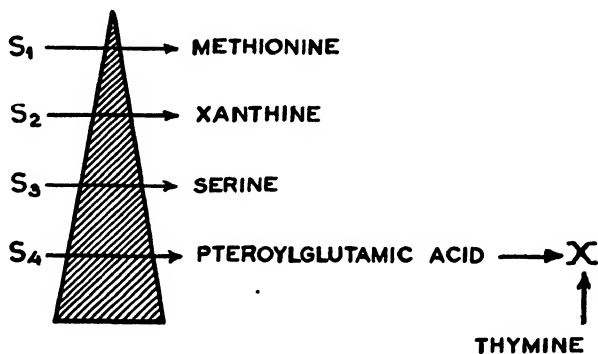

FIG. 5. Diagram of the reaction supposed to be inhibited by sulfanilamide.

ing concentrations of sulfanilamide (shaded area). The synthesis of methionine (blocked by small concentrations) is the most sensitive to SA and, the synthesis of pteroylglutamic acid is the least sensitive of the series.

As, in the presence of the other 3 antagonists, thymine and pteroyl-glutamic acid are both active independently, they evidently represent alternative pathways to the same end-product. There are then 3 possibilities: a. the end-product is pteroylglutamic acid, thymine an intermediate; b. the end-product is thymine, pteroylglutamic acid an intermediate; c. the end-product is an unknown substance X, which can be produced from both intermediates thymine and pteroylglutamic acid. The first possibility is highly improbable. In *Streptobacterium plantarum* and *L. arabinosus* the synthesis of pteroylglutamic acid from PABA is inhibited by SA. A synthesis of pteroylglutamic acid by *B. coli* from endogenous PABA and thymine would be blocked equally well by SA.

The second possibility is supported by the experiments of Stokes (16), who showed that thymine could substitute pteroylglutamic acid in the growth of some species of *Lactobacilli*.

We favor the third possibility as pteroylglutamic acid might easily have another function apart from catalyzing thymine production, particularly since the addition of thymine *and* pteroylglutamic acid in our experiments permitted growth in shorter times than with thymine alone.

Shive (14) started from the supposition that the end-products of the inhibited reactions all arise from the same substrate, building his theory on the structural relationship between inhibitor and substrate. He soon included the supposition of different substrates with a common catalyst, structurally related to the inhibitor, which covers the facts equally well. On the submitted facts it is, of course, impossible to decide between the two assumptions. The argument that 35 mg. of methionine cannot be produced from some γ of PABA might support the view that different substrates are involved. The high concentration or pteroylglutamic acid which is necessary for growth seems equally to preclude its synthesis from PABA. One should distrust this kind of reasoning, however, as the concentration inside the bacteria might be much smaller. What was said above about the possibility of alternative metabolic pathways invalidates these arguments still further. Whether PABA plays the rôle of a substrate or a catalyst, therefore, remains undecided. We favor the latter view, however.

As growth in the presence of 2000 mg./l. SA is restored (without PABA) by the 4 antagonists (m, x, s, and thymine, or m, x, s, and pteroyl-glutamic acid), one might argue that the set of noncompetitive anta-

gonists for *E. coli* was herewith complete. This cannot be quite true, as full growth is only obtained in about 72 hours, (with SA and optimal concentration of PABA about 32 hours) whereas in the control (without SA) full growth occurs in 24 hours. There are two possibilities to explain this fact.

(a) Still further enzyme-reactions are blocked by SA, but incompletely, growth thus being retarded, not inhibited.
(b) The antagonists are permitting growth by alternative pathways, with slower reaction velocities.

We are inclined to favor the second view, as the addition of a further antagonist in the series never affected the velocity of growth.*

SUMMARY

With *E. coli* on a synthetic medium it was shown that with increasing sulfanilamide concentrations 4 enzyme systems become inhibited successively.

Methionine, xanthine, serine, and pteroylglutamic acid (in this order) are the noncompetitive antagonists of the inhibited reactions. Pteroylglutamic acid is a noncompetitive antagonist only in the presence of methionine, xanthine, and serine. With these 4 substances growth is obtained in the presence of 2000 mg./l. sulfanilamide. Thymine can substitute pteroylglutamic acid. With *Salmonella typhimurium* similar results were obtained.

* *Note added in proof.* Further experiments showed that normal growth in the absence of PABA was obtained in about 30 hours, when valine was added to the medium; lysine or isoleucine were active to a lesser extent. Valine might thus be regarded as the end-product of a further enzyme system inhibited by sulfanilamide.

With several sulfanilamide compounds, *e.g.*, sulfathiazole, sulfapyridine, 5-sulfa-2-methylpyrimidine the same series of noncompetitive antagonists, including valine, was found. The sulfanilamide concentrations by which each system was inhibited were, however, crowded together in a much narrower range of concentrations. With sulfanilic acid the inhibition of the postulated enzyme systems is again spread out over a wider range of concentrations. Adsorption phenomena probably explain these facts.

With regard to the methionine system, neither sodium sulfide, cysteine, homocysteine nor a mixture of homocysteine + choline could replace methionine. One might suppose an inhibition of the methylation of homocysteine by sulfanilamide.

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On the Fermentability of Fructofuranose

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INTRODUCTION

The selective fermentation of glucose-fructose mixtures by living yeasts has been known for a long time (*cf.* 1, 9a). In such a mixture, most yeasts, as, *e.g.*, bakers' and brewers' yeasts, selectively prefer glucose. On the other hand, when the sugars are in separate solutions, fructose is fermented at the same rate as glucose over a wide range of concentrations (*cf.* only 26). However, this equality of the rates of fermentation has been confirmed by Hopkins and Roberts (14) only in concentrations above 1%. Below this limit fructose is fermented—by brewers' yeast—more slowly than glucose, the rate of fermentation of fructose, at a concentration of 0.1% and 30°C., being only 63% that of glucose.

Hopkins (13, 15,16) bases his explanation of this phenomenon on the specificity of most yeasts for the normal pyranose forms of aldohexoses, glucose and mannose, but for the furanose form of fructose (γ-fructose); hexoses derived directly, *e.g.*, from maltose and sucrose, are specifically fermented in this way. In this connection, Hopkins calls attention to the reciprocity of Haworth's projectional formulae of glucopyranose, mannopyranose and fructofuranose, as compared to fructopyranose.

Since fructose, like other hexoses, occurs in solution chiefly in the pyranose form, the amount of the fermentable furanose modification at equilibrium with the pyranose form remains rather small. Fructose would thus be taken up more slowly in fermentation of a mixture of glucose and fructose, or at low concentrations. The conversion rate of fructopyranose to the furanose form must thus be considered a limiting factor in the fermentation of fructose.

This explanation of Hopkins' rests on the assumption that only the pyranose and furanose forms of normal fructose exist in an aqueous solution. This view has been confirmed by Isbell and Pigman (19). In addition, it is also indicated by investigations on sorbose (23, 22), which show that the α–β interconversion occurs in the pyranose ketose series but that the equilibrium lies far to one side. It is true that a fructose

solution at equilibrium also contains the open chain carbonyl modification but, according to Isbell and Pigman, in minute concentrations only.[1]

Crystalline fructose exists exclusively in the pyranose form. When dissolved, part of this fructopyranose rapidly mutarotates to the furanose form. As heat is absorbed in this mutarotation of freshly dissolved fructose, the energy content of the pyranose form must be less than that of the labile furanose modification. This agrees with the fact that, at a higher temperature, the equilibrium lies more toward the side of the furanose form than at a lower one (19).

According to Haworth it is now generally believed that the fructose constituent of sucrose is a furanose. Hudson (17, 18) has already shown that, when sucrose is split by saccharase, the form of fructose liberated mutarotates at a rate equal to that of solid fructose when dissolved, but in the opposite direction. The fructofuranose, split off by saccharase, is then stabilized into the pyranose form. This conversion is rapid (24, 19). Accordingly, Hartley and Linnell (10) have calculated that the half-life period of the furanose form of fructose (γ-fructose)—split off by saccharase at pH 4.64—would be 7½ mins. at 15°C., and only 3 mins. at 25°C. (cf. also 7). These figures correspond to a total life period of the furanose form of approximately 75 mins. and 30 mins., respectively.

However, when sucrose is fermented by yeast under conditions involving *continuous* inversion, the furanose form of fructose would be present in excess in the immediate vicinity of the yeast cell. Insufficient quantity of the furanose form, then, should not be a limiting factor in the fermentation of fructose. A comparison of the fermentation of such a glucose-fructose mixture *in statu nascendi* to the fermentation of a corresponding solution of invert sugar—a stabilized mixture of glucose and fructose—would thus be a new starting point for studying the fermentability of fructofuranose and the selective fermentation of glucose and fructose.

Recently Gottschalk (4) reports having confirmed the deduction that fructofuranose represents the fermentable fructose modification by a comparison of the fermentation rates with yeast at 0°C. using fructopyranose, equilibrium fructose, and fructofuranose (set free from sucrose) as substrates. Subsequently, he has mentioned (7, 8), in addition, that, when sucrose is fermented by bakers' yeast at 0°C. and at pH 4.5— under circumstances in which the mutarotation of fructose proceeds at its minimum rate—approximately equal amounts of glucose and fructose are left after fermentation periods of 20 or 40 mins. in contrast to the preponderance of fructose in the residual aldose-ketose mixture when sucrose is fermented at 25°C.

EXPERIMENTAL

Material

The bakers' yeast used was produced by the Rajamäen Tehtaat (Rajamäki Factories) of Oy Alkoholiliike Ab, Helsinki. The brewers' yeast originated from Ab P. Sinebrychoff Oy, Helsinki.

The sucrose employed was a very pure commercial product (99.85%). From this the invert sugar solution was prepared by adding 3 ml. Merck's "Invertin"/l. of 10%

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[1] According to Sørensen (27), however, fructose at equilibrium consists to a considerable extent of the open chain form, that is, about 1% of the total fructose present.

sucrose solution and allowing the solution to stand over night at 30°C. During this time the sucrose was completely inverted.

Experimental Procedure

A weighed amount of yeast (10–40 g.) was suspended in 100 ml. tap water. The suspension was filtered on a ϕ 7 cm. Büchner funnel through Schleicher & Schüll No. 597 filter paper. The slightly turbid filtrate was poured back into the funnel and a filter paper placed on the surface of the liquid, after which the liquid was drawn off. When brewers' yeast was employed, it was found sufficient to filter the suspension only once, the filter paper being placed on the surface right at the start. The purpose of this filter paper which, after drawing off the liquid, remains on top of the yeast layer, is partly to protect the yeast from drying and partly to prevent its becoming mixed with the sugar solution which is later poured on the yeast.

This "yeast filter" was now connected to a suction flask connected in turn to another suction flask containing water. The passage time was determined by drawing 100 ml. tap water through the "yeast filter." A 50 ml. graduated micro Kjeldahl flask was used as an adapter in the suction flask.

The 15°C. sugar solution (1–3%) was poured into the funnel and a definite quantity (42 ml.) drawn through the filter, the passage time being simultaneously noted. Since there was reason to expect that during the initial phases of the experiment— when the yeast "takes up" sugar from the solution—the results might differ from those in the later phases, a new sample of equal volume was now taken into another adapter. Sugar determinations from the filtrate were made immediately after taking the samples.

The purpose of the "yeast filter" was—by allowing only a brief contact of the sucrose and yeast—to prevent as far as possible a premature inversion of sucrose so that the liberated fructofuranose would not have time to convert into the pyranose form. It could be proved, indeed, that even during a prolonged filtration there was no inversion in the solution *above* the filter paper. This is seen from the following experiment:

The "yeast filter" was prepared from 25 g. brewers' yeast. 42 ml. of 1% sucrose solution was filtered through the funnel in 21 mins. Samples drawn from the solution above the filter paper showed no inversion; when hydrolyzed with dilute hydrochloric acid according to Herzfeld and Dammüller (11) the invert sugar content was found to be 98.4%.

When larger quantities of yeast were employed, the passage time of the sugar solution became so long that the possibility of a conversion of the fructofuranose could not be completely eliminated. Hartley and Linnell (10) have calculated the half-life period of fructofuranose at 15°C. to be only 7½ mins. For this reason, a series of experiments were set up with the same technique but using a ϕ 12.5 cm. Büchner funnel with 40 g. yeast. A sufficient quantity of filtrate could then be obtained in 2–3 mins., whereby the danger of a stabilization of fructofuranose became practically negligible.

The above "yeast filter" method could probably be successfully employed generally for the selective removal of fermentable sugars from solutions, for instance, for the determination of fermentable sugars in solutions containing other reducing sub-

stances (blood, wood hydrolyzates), and for the removal of small amounts of glucose from certain sugar preparations, such as galactose and fructose.

Analytical

Most investigations on the selective fermentation of glucose and fructose mixtures are made by following the change of optical rotation of the sugar solutions during the fermentation. Only Schiller (25), Fernbach *et al.* (3) and Hopkins (12) have, in comparative experiments, determined glucose by the iodine oxidation method, according to the principle of Willstätter and Schudel; fructose was then calculated from the difference between total reducing sugar and glucose. This method has recently been employed also by Gottschalk (7).

TABLE I

Loss of Glucose and Fructose on Passage of 1% Invert Sugar
Solution through the "Yeast Filter" at 15°C.

Yeast strain	"Yeast filter" ϕ 7 cm.		Filtered sugar solution					
	Quantity of yeast	Passage time of water	Fraction (42 ml.) no.	Passage time	Invert sugar	Glucose	Fructose	$\dfrac{\text{Glucose}}{\text{Fructose}}$
	g.	*mins.*		*mins.*	*mg./ml.*	*mg./ml.*	*mg./ml.*	
Bakers'	10	20	1	6	181.8	87.2	94.7	0.92
			2	12	191.6	89.6	101.9	0.88
	25	30	1	11	134.7	59.4	75.4	0.79
			2	23	154.3	59.6	94.7	0.63
	25	30	1	15	148.0	66.6	81.4	0.82
			2	30	123.0	38.8	84.1	0.46
	40	48	1	18	85.4	19.4	66.0	0.29
			2	36	82.0	4.8	77.2	0.07
Brewers'	10	22	1	10	167.5	76.4	91.2	0.84
			2	20	174.7	77.2	97.5	0.79
	25	34	1	17	125.1	47.6	77.5	0.61
			2	30	121.6	35.7	85.9	0.42
	25	43	1	19	103.6	31.3	72.3	0.43
			2	37	96.1	9.8	86.3	0.11
	40	53	1	25	40.6	0.0	40.6	0.00
			2	54	54.6	0.1	54.5	0.00

TABLE II

Loss of Glucose and Fructose on Passage of 1% Sucrose Solution through the "Yeast Filter" at 15°C.

Yeast strain	"Yeast filter" φ 7 cm.		Filtered sugar solution					
	Quantity of yeast	Passage time of water	Fraction (42 ml.) no.	Passage time	Invert sugar	Glucose	Fructose	Glucose / Fructose
	g.	*mins.*		*mins.*	*mg./ml.*	*mg./ml.*	*mg./ml.*	
Bakers'	10	18	1	6	182.7	90.1	92.6	0.97
			2	14	186.5	90.0	96.5	0.93
	25	30	1	13	144.4	70.8	73.6	0.96
			2	25	155.8	75.9	79.9	0.95
	40	43	1	21	104.6	51.4	53.3	0.96
			2	38	91.5	42.8	48.7	0.88
Brewers'	10	19	1	10	160.6	78.7	81.8	0.96
			2	18	177.7	88.0	89.7	0.98
	25	30	1	12	117.2	57.5	59.6	0.96
			2	25	127.4	62.9	64.5	0.97
	25	30	1	13	119.2	60.0	59.2	1.01
			2	25	120.6	60.3	60.3	1.00

TABLE III

Loss of Glucose and Fructose on Rapid Passage of 1% Invert Sugar Solution through the "Yeast Filter" at 15°C.

Yeast strain	"Yeast filter" φ 12.5 cm.	Passage		Filtered sugar solution			
	Quantity of yeast	Time	Volume	Invert sugar	Glucose	Fructose	Glucose / Fructose
	g.	*mins.*	*ml.*	*mg./ml.*	*mg./ml.*	*mg./ml.*	
Bakers'	40	3	38	102.3	49.4	52.9	0.93
Brewers'	40	3	—	119.7	55.9	63.8	0.88
	40	3	28	69.8	33.4	36.3	0.92

TABLE IV

Loss of Glucose and Fructose on Rapid Passage of 1% Sucrose
Solution through the "Yeast Filter" at 15°C.

Yeast strain	"Yeast filter" φ 12.5 cm.		Filtered sugar solution			
	Quantity of yeast	Passage		Invert sugar	Glucose	Fructose	$\dfrac{\text{Glucose}}{\text{Fructose}}$
		Time	Volume				
	g.	*mins.*	*ml.*	*mg./ml.*	*mg./ml.*	*mg./ml.*	
Bakers'	40	3	25	89.2	45.9	43.3	1.06
Brewers'	40	3	—	94.4	49.2	45.2	1.09
	40[a]	2	15	74.4	38.5	35.9	1.07

[a] 3% sucrose solution.

We found the iodine determination of glucose unsatisfactory, since the fermentation solution also contains other iodine-reducing substances (*cf.* 12, 7). The method of Kolthoff-Kruisheer (20, 21) was therefore applied to the determination of glucose in the presence of fructose, and found to give reliable results. Thus, the mean value for the ratio glucose/fructose in our invert sugar from different determinations was 0.99, while the recovery of invert sugar varied from 97.5 to 98.0% (on fructose basis 97.8–98.6%).

The analysis of the fermentation liquid was carried out as follows: 20 ml. of the filtered sample were pipetted into a 100 ml. measuring flask, containing 50 ml. 0.1 N NaOH—to stop the fermentation—and the flask filled to the mark. Invert sugar was determined from an aliquot of 20 ml. according to Schoorl. That the inversion of sucrose by yeast had been complete, was controlled separately, by hydrolyzing with dilute HCl according to Herzfeld and Dammüller (11). Another sample of 20 ml. was pipetted into a 50 ml. measuring flask, and 1.25 ml. of 4 N NaOH was added. In this sample glucose was destroyed according to Kruisheer using, however, only ⅓ of the amount of solutions. The solution was then made up to the mark and reducing sugar—fructose—determined on an aliquot of 20 ml. according to Schoorl. Glucose was then calculated from the difference between total invert sugar and fructose.

The results are compiled in. Tables I–IV.

DISCUSSION

It was mentioned above that, according to Hopkins, brewers' yeast as well as bakers' yeast specifically ferment one form of fructose in a solution at dynamic equilibrium, as evidenced by the positive mutarotation of the partially fermented fructose solution. He assumes this fermentable form to be fructofuranose, which shows certain features

of configuration common to glucopyranose and mannopyranose. The velocity of mutarotation of the pyranose modification to replace the fructofuranose at equilibrium would thus be the limiting factor for the fermenting velocity of fructose.

There are weighty arguments in favor of Hopkins' view that the high-energy furanose form of fructose is fermentable. In low concentrations, when the absolute amount of the furanose form in solution is low, fructose is fermented much more slowly than glucose (13, 14). In higher concentrations again there is a proportionality between the amount of yeast and the rate of fermentation, up to a certain yeast concentration. Above this limit, the rate of fermentation is less than proportional, *i.e.*, the furanose form is evidently present in insufficient quantities (14). According to Gottschalk (5, 8), at 0°C. a solution at equilibrium contains about 12% of fructose in the furanose form. A rise in temperature shifts the equilibrium toward the furanose form so that at 20°C. 22% appears as the furanose modification. Likewise, the concentration of the fermentable component of fructose in solution also increases (12, 13). Thus, the temperature coefficient of fermentation for a 0.25% fructose solution is in close agreement with the temperature coefficient of conversion of fructopyranose to equilibrium (15).

To decide whether fructopyranose is fermentable by yeast, Gottschalk (4) has recently made use of the fact that mutarotation of fructose proceeds at its minimum rate at 0°C. and at pH values between 3.2 and 5.1. The rate of fermentation of fructose, added in the crystalline state to a bakers' yeast suspension under these conditions, was almost identical with the rate of mutarotation by which part of the pyranose form is converted into furanose. This minute rate of furanose consumption was—in contrast to the rate of glucose consumption—independent of yeast concentration, indicating the non-enzymatic nature of the rate-determining factor. In addition, at 25°C. and low substrate concentration, increase in yeast concentration does not result in a proportional increase in the rate of fructose fermentation, whereas this is the case with glucose as substrate (9). From these results he concludes that fructopyranose is unfermentable by yeast, while fructofuranose represents the fermentable modification.

Our results, using a special technique, also indicate that in fermentations by bakers' and brewers' yeast the furanose form of fructose is preferred to the pyranose form. It is especially to be noted that when sucrose is fermented—and the conversion of fructofuranose into the pyranose form is prevented by using short experimental times— fructose has been fermented more rapidly than glucose (*cf.* also 6, 8). On the inversion of sucrose (α-glucopyranosido-β-fructofuranoside) its

glucose component is split off in the α-modification and is thus fermented without any preliminary change of its molecular structure (28, 16, 4). Since the α-β interconversion of glucose proceeds at a very much slower rate than the mutarotation of fructose, the above observations prove that *fructofuranose in reality is fermented more readily than α-glucose, which has been considered the most rapidly-fermented hexose modification.*

The ready fermentability of fructofuranose will explain the fact that sucrose is fermented by yeast faster than invert sugar. However, since the inversion of sucrose by yeast proceeds much more rapidly than the fermentation, the faster fermentation of sucrose in regard to invert sugar would be limited to the initial stages of fermentation, at least with more concentrated solutions, because the hexose mixture is stabilized into invert sugar as soon as the inversion of sucrose is completed. This view is fully confirmed by Cohn's (2) work, both with top yeast and with bottom yeast.

In the technical production of bakers' yeast by the "Zulauf"-method the sugar content of the growth solution is kept not only as constant as possible but also considerably low. When sugar is being added continuously and in small quantities only, it is immediately utilized by yeast. Under these conditions there is hardly time for the fructose, which is split off from sucrose of molasses, to stabilize into the pyranose form, but at least the major part of it will be utilized in the high-energy furanose modification. This circumstance may also be conducive to the increase of yeast yield obtained by the "Zulauf"-method.

The fact that the *high-energy* furanose form of fructose is preferred by yeast, does not yet indicate the exact form in which fructose is being fermented. It is true that Gottschalk (4, 8, 9) interprets the nonfermentability of fructopyranose by the lack of an OH-group attached to carbon atom 6, because the presence of this group—as in fructofuranose—is considered essential for fermentation. However, it can just as well be conceived that, for fermentation, fructose must convert into any other form and that this conversion takes place most readily in the furanose modification owing to its high energy content.

Our experiments, performed without destroying the yeast cells, seem to indicate that, *in sugar fermentations, the selection occurs at the cell surface and not within the cells.* This view is, however, in sharp

contrast to the prevailing opinion that all components of the sugar in question diffuse into the cell and that the removal of the fermentable sugar and its replacement in restored equilibrium takes place within the cell, controlled by the reaction of highly buffered cell contents (15, 4; *cf.*, however, 9, 9a).

SUMMARY

The rate of fermentation of fructofuranose by living bakers' and brewers' yeast was studied *with a new technique* involving the use of "yeast filter."

By this technique it is possible to observe immediately the changes which take place in the fermenting solution, outside the yeast cells. The fact that large quantities of yeast come into only a passing contact with a comparatively weak sugar solution, facilitates the detection of changes in the fermenting solution. By using low temperatures and brief experimental periods the conversion of the fructofuranose—split off from sucrose—into the pyranose form could be prevented.

When the substrate was invert sugar—an equimolar glucose-fructose mixture at equilibrium—glucose disappeared from the solution more rapidly than fructose at 15°C. With longer experimental periods, under conditions corresponding to those of selective fermentation, practically glucose-free fructose solutions were thus obtained. Even when the experimental periods were as brief as possible, glucose was fermented faster than fructose, the ratio glucose/fructose varying from 0.88 to 0.93. When the substrate was sucrose—*i.e.*, an equimolar glucose-fructose mixture *in statu nascendi*—the rate of fermentation of fructose at 15°C. was nearly the same as that of glucose, and, even with longer experimental periods, the ratio glucose/fructose rarely decreased below 0.90. With brief experimental periods, fructose definitely disappeared from the solution faster than glucose, the ratio glucose/fructose varying from 1.06 to 1.09.

In view of the fact that, on inversion of sucrose, fructose is set free in the furanose form, these results support the view that the high-energy furanose form is preferred in yeast fermentations. The results also show that fructofuranose is fermented both by bakers' yeast and by brewers' yeast more readily than α-glucose which has been regarded as the most readily-fermented hexose modification.

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The Effects of Pteroylglutamic Acid and of Xanthopterin on Reproduction and Lactation in the Rat [1]

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INTRODUCTION

In their studies on reproduction and lactation in the rat, Cerecedo and Vinson (1) observed that concentrates of folic acid and of an *L. casei* factor, used in small quantities, improved their basal diet so that litter size was larger, weaning weights were higher, and the strain on the mother was lessened. Because of the limited amount of material available to them, these workers fed the concentrates during pregnancy and lactation only. In a similar study, Richardson and Hogan (2) observed a beneficial effect on the percentage of young weaned when 1% of a Fuller's earth eluate of liver containing vitamin B_c was added to their synthetic diet from weaning. A similar effect was produced by 5% of a liver filtrate which contained only 0.3 γ of vitamin B_c/g. These authors, therefore, were not able to draw any definite conclusions from their experiments regarding the beneficial effect of folic acid. Nelson, van Nouhuys and Evans (3), using a short-term experiment similar to that used by Hartwell (4), and by Daggs and Tamboulian (5), reported no beneficial effect on lactation from small quantities of a folic acid concentrate. Following up these experiments, Nelson and Evans (6) supplemented their basal diet with a liver extract assaying 550 γ of *L. casei* factor/g., and found that it improved the basal ration to the extent that weaning weights of the young, and weight changes of the nursing mothers, were only slightly below normal. However, these workers were not certain that folic acid was

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beneficial because a concentrate had been used. In a later study (7), by supplementing the basal diet with pteroylglutamic acid in amounts of 1.1, 2.75 and 5.5 mg./kg., they found that the lactating rat requires 33 γ of this vitamin per day for maximal effect on the maternal body weight, and 33–81 γ per day for maximal effect on the weaning weights of the young.

The present study was undertaken to clarify and extend some of the previous work on pteroylglutamic acid. In addition, it was deemed of interest to study the effect of xanthopterin because of its chemical and physiological relationship to pteroylglutamic acid.

EXPERIMENTAL

In this study, rats of the Wistar, Sprague-Dawley, and Long-Evans strains were used. Since no significant strain differences were found, the results with the 3 strains have been grouped together. The basal diet used, diet R-5a (Table I), is a slight modification of that developed by Vinson and Cerecedo (8). Supplements were added to this basal diet from weaning or during pregnancy and lactation only.

TABLE I
Composition of Diet R-5a

	per Kg.
Purified casein[a]	300 g.
Sucrose	480 g.
Hydrogenated vegetable oil[b]	100 g.
Lard	50 g.
Ruffex[c]	20 g.
Salts[d]	50 g.
Thiamine chloride	20 mg.
Riboflavin	20 mg.
Pyridoxine hydrochloride	20 mg.
Calcium pantothenate	40 mg.
Choline chloride	500 mg.
α-Tocopherol	20 mg.
Vitamin A concentrate[e]	67.5 mg.
Vitamin D [f]	5000 I. U.

[a] Labco or Smaco.
[b] Crisco or Primex.
[c] Purified roughage material consisting of processed, vitamin-free rice hulls. Equivalent to 70 % α-cellulose (balance simple- and hydrocelluloses).
[d] Osborne-Mendel salt mixture. The quantity of manganese was doubled.
[e] Equivalent to 67,500 I. U.
[f] Given in the form of Drisdol (Winthrop).

Pteroylglutamic acid [2] was fed at three levels—5, 10 and 20 mg./kg. of diet—during pregnancy and lactation only, and at two levels—10 and 20 mg./kg. of diet—from weaning. Since little difference was found in the results with the various levels in each group, the data have been combined for the purposes of this paper.

A limited amount of xanthopterin [3] was available. This was fed at 5 and 10 mg. levels (per kg. of diet) during pregnancy and lactation, and at a 10 mg. level from weaning.

Stock controls were fed Purina [4] and Rockland [5] diets.

Litters were reduced to 6 young on the third day of lactation (at two days of age). Since, in our experience, many litters die or are destroyed during the first two days due to inherent weakness at birth (9), only those litters and young surviving on the third day are included in the lactation results. In calculating average weaning weights of young and average weight change of the mothers, only litters in which 5 or 6 young were weaned have been included. "Reproduction success" represents the percentage of pregnancies resulting in living young on the third day of lactation.

Female rats were mated at 3 months of age with stock males. After a successful lactation, a rest period of 3 weeks was given before re-mating. If lactation was unsuccessful, a rest period of 2 weeks was given.

RESULTS AND DISCUSSION

The results obtained have been summarized in Tables II and III.

The previous findings of Cerecedo and Vinson (1), concerning the beneficial effect of folic acid concentrates on the weaning weights of the young and on the weight change of the mother when given during gestation and lactation only, have been confirmed. We have also confirmed by a long-term experiment the improvement in lactation found by Nelson and Evans (7) on feeding pteroylglutamic acid (PGA) in a short-term test.

[2] We are indebted to Drs. B. L. Hutchings and E. L. R. Stokstad of the Lederle Laboratories for this material.

[3] Kindly supplied by Dr. W. A. Lott of the Squibb Laboratories and Dr. T. H. Jukes of the Lederle Laboratories.

[4] A commercial stock diet containing the following: Meat meal, dried skimmed milk, wheat germ, fish meal, liver meal, dried beet pulp, corn grits, oat middlings, soybean oil meal, dehydrated alfalfa meal, molasses, riboflavin supplement, brewers' dried yeast, thiamine, niacin, vitamin A and D feeding oils, D activated plant sterol, 1% steamed bone meal, 0.5% iodized salt, and 0.02% manganese sulfate.

[5] A commercial stock diet containing the following: Cane molasses, soybean oil meal, animal liver meal, fish meal, meat scraps, condensed buttermilk, corn gluten meal, irradiated brewers' type yeast, wheat germ oil, O. P. linseed oil meal, corn oil meal, ground oats, wheat bran, wheat flour midds, ground yellow corn, ground hulled barley, ground hulled oats, ground whole wheat, whole milk powder, alfalfa leaf meal, vitamin A oil, 0.5% steamed bone meal, 1% calcium carbonate from limestone, and 2% salt.

TABLE II

The Effects of Pteroylglutamic Acid and of Xanthopterin on Reproduction

Diet	Number of rats	No. of pregnancies	No. of litters born alive	No. of living young	No. of dead young	Number of living young per litter	Number of dead young per litter	Litters alive—third day	Reproduction success
									Per cent
Stock	71	131	125	1042	54	8.3	0.4	113	86.3
R-5a	50	79	57	385	56	6.8	1.0	28	35.4
PGA (given during gestation and lactation)	19	30	28	193	30	6.9	1.1	15	50.0
PGA (given from weaning)	14	24	19	141	12	7.4	0.6	17	70.8
Xanthopterin (given during gestation and lactation)	28	47	34	211	24	6.2	0.7	18	38.3
Xanthopterin (given from weaning)	9	17	14	86	29	5.1	1.7	5	29.4

TABLE III

The Effects of Pteroylglutamic Acid and of Xanthopterin on Lactation

Diet	Litters alive—third day	Litters weaned	Litters weaned	Young given for nursing	Young weaned	Young weaned	Average weaning weight of young	Average weight change in mothers	Average number weaned	Ratio of litters weaned to pregnancies
			Per cent			*Per cent*				
Stock	113	105	92.9	678	617	91.0	39.1	– 3.8	5.9	0.80
R-5a	28	20	71.4	156	107	68.6	36.5	–26.7	5.4	0.25
PGA (given during gestation and lactation)	15	8	53.3	74	38	51.4	39.7	– 6.8	4.8	0.27
PGA (given from weaning)	17	14	82.4	91	76	83.5	35.4	– 4.6	5.4	0.58
Xanthopterin (given during gestation and lactation)	18	14	77.8	94	75	79.8	39.2	–18.8	5.4	0.30
Xanthopterin (given from weaning)	5	5	100.0	26	26	100.0	36.9	– 8.0	5.2	0.29

In our experiments, the feeding of PGA during gestation and lactation only exerted a beneficial effect chiefly on reproduction as evidenced by the greater percentage of litters alive on the third day. A considerable number of litters have died or been destroyed after the third day which is reflected in the poor figures for the percentage of young and litters weaned. In surviving litters, however, the weights of the young were good and the strain on the mother was lessened considerably. The overall picture, as shown by the ratio of litters weaned to pregnancies, is not markedly different from that obtained with the basal diet.

When PGA was given from weaning, a beneficial effect was found on both reproduction and lactation. In the case of reproduction, this is observed in the number of living young per litter and in the percentage of litters alive on the third day. This latter criterion indicates our belief that neonatal deaths should be included with the other manifestations of reproductive failure. Lactation was also improved in certain respects. An exception is found in the case of the average weaning weight of the young. A similar condition is found when xanthopterin is fed from weaning. We believe that this may be closely connected with the fact that a higher percentage of young and litters are weaned.

Xanthopterin appears to have caused no improvement in reproduction when given either from weaning or for the limited period. A beneficial effect was found on lactation, which was somewhat greater when the supplement was given from weaning, except as stated above. It should be mentioned here that xanthopterin has been found to cause a transitory stimulating effect on the blood picture in vitamin M-deficient monkeys (10), and to alleviate the leucopenia and counteract the growth inhibition produced in the rat by succinylsulfathiazole (11). Mirone and Cerecedo (12) have recently reported a beneficial effect of xanthopterin on lactation in the mouse.

Since the feeding of PGA from weaning appeared to promote improved reproduction and lactation, it occurred to us that PGA is required not only during pregnancy and lactation, but also during the period of growth, to insure more optimal conditions for gestation and lactation. While this was not reflected in an additional gain in weight of the mothers during the growth period, it seemed possible that other physiological functions (13) might be aided in this manner.

Our experiments with PGA lead us to question the advisability of using only short-term experiments in lactation studies. Such studies are undoubtedly of value in comparing diets, but, in our opinion, they

are of limited value in evaluating the adequacy of a synthetic diet which is to be used for long-term experiments through several generations. We believe that so-called "reproduction factors" and "lactation factors" act by promoting the well-being of the animal rather than by a specific effect on these functions. Such "factors" would be expected to exert at least the same beneficial effect when given for the limited period as when given from weaning. On this basis, the effect of a synthetic diet on reproduction and lactation is best expressed by calculating an overall ratio, that is, a ratio of litters weaned to pregnancies Such data are given in Table III.

SUMMARY

1. The beneficial effect of pteroylglutamic acid on reproduction and lactation in the rat has been confirmed. This vitamin is not considered a "lactation factor," however, since it is decidedly more beneficial when added to a synthetic diet from weaning than when given during pregnancy and lactation only.

2. Xanthopterin was found to have some beneficial effect on lactation in the rat.

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The Microbiological Assay of Extracts Containing Toxic Materials

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When interpreting the results of a microbiological assay, the increase in growth of an organism, upon addition of aliquots of the extract being assayed, is assumed to be due solely to the substance being assayed which is present in each of these aliquots. When the test organism is grown on a medium which is nutritionally optimum except for the substance being assayed, and when relatively small aliquots of the extract are added to the medium, such an assumption is usually valid, or sufficiently so for the purpose in hand. Many natural extracts, however, contain substances which inhibit the growth of the test organism, and when large aliquots of these toxic extracts must be used (because of a low content of the substance being assayed) the growth response of the organism is decreased below that which one would obtain if the toxicity were not present. The presence of toxic materials in an extract is usually quite clearly indicated in a microbiological assay by the fact that the apparent content of the assayed substance becomes smaller when calculated from assays of larger aliquots of the extract.

The problem of obtaining a satisfactory assay on such toxic extracts has been approached in 3 general ways: (1) by decreasing the size of the aliquot assayed to the point where toxic effects are no longer significant, (2) by a preliminary separation of the toxic materials from the extract, and (3) by supplementing the medium with a large excess of the toxic materials so that the amount added with the aliquot to be assayed will not be significant.

The first of these methods is the one most widely employed. It is, of course, no real solution to the problem, since it presupposes an ability to "dilute out" the toxic effect while still retaining sufficient growth-stimulatory effect from the substance being assayed to obtain a measurable and statistically significant growth response. This is

not possible in the case of many "low potency" extracts. Nevertheless, much data has found its way into the literature which is based upon the assay of samples containing so little of the substance being assayed that the maximum growth response measured is equivalent to only a few per cent of the total range of the standard growth response curve. Little quantitative significance can be attached to data obtained in such a fashion.

The second method is the ideal solution to the problem, but its application may be tedious or, in many instances, impossible. For the application of this method a procedure must be available whereby the toxic materials can be removed without altering the substance to be assayed. If such a method is not available it may be possible to destroy the substance to be assayed without affecting the toxicity. In this event the third method may be employed. Supplementing the assay medium with a large excess of the toxic materials is a none too satisfactory solution of the problem, however, since it cannot safely be assumed that on such a drastically non-optimum medium the growth of the test organism will depend solely on the substance being assayed. This method has the further disadvantage of requiring the preparation of a separate "toxic supplement" from each sample or type of sample which is to be assayed.

In the course of studies on the amino acid content of urine, this problem was encountered and a method devised which offers many advantages over those reviewed above. In essence the method consists of determining the standard growth response to the substance being assayed, in the presence of the same concentration of toxic substances as are present in the extract being assayed. The procedure is best illustrated by the example shown in Table I.

A consideration of the calculations outlined in Table I will reveal that, if they are to be considered valid, two conditions must be met: (1) the measured growth response of the test organism in the presence of a constant amount of inhibitory materials must be a linear function of the amount of assayed substance present, and (2) the growth in the blank must not be affected by the addition of inhibitory substances. The second of these conditions can be met only if the blank shows essentially no growth, and the method outlined in Table I is not applicable where this is not the case. In most of the well-developed microbiological assay procedures, however, little or no growth occurs in the blanks.

Concerning the first condition it has been our experience, using a wide variety of microbiological assay methods and measuring the growth response turbidimetrically, that by choosing a suitable function of the measured turbidity, a linear response can nearly always be obtained over at least the first 40–50% of the total range of growth. We have used a thermoelectric turbidimeter (1) for our measurements, and the galvanometer readings, plotted directly, usually result in a suitably linear relationship.

TABLE I

Assay of Toxic Urine Sample for Lysine

Tube no.	Tube contents in addition to basal medium plus water to 5 ml. volume	Growth response (turbidity)[a]
0	nothing ("blank")	4.5
1	0.2 ml. urine	12.5
2	0.2 ml. urine plus 6γ lysine	18.0
3	0.2 ml. urine plus 12 γ lysine	23.0
4	0.4 ml. urine	18.5
5	0.4 ml. urine plus 6 γ lysine	23.0
6	0.4 ml. urine plus 12 γ lysine	27.5
7	0.6 ml. urine	24.0
8	0.6 ml. urine plus 6 γ lysine	28.5
9	0.6 ml. urine plus 12 γ lysine	32.5

Calculations based on tubes 1, 2 and 3:

Response due to 0.2 ml. urine $= 12.5 - 4.5 = 8.0$

Response due to 6 γ lysine (tube 2) $= 18.0 - 12.5 = 5.5$

Response due to 6 γ lysine (tube 3) $= 23.0 - 18.0 = 5.0$

Average response due to 6 γ lysine $= (5.5 + 5.0)/2 = 5.25$

Lysine content of urine $= 6 (8.0/5.25) = 9.15$ γ/0.2 ml. $= 46$ γ/ml.

Calculations based on tubes 4, 5, and 6:

Response due to 0.4 ml. urine $= 18.5 - 4.5 = 14.0$

Response due to 6 γ lysine (tube 5) $= 23.0 - 18.5 = 4.5$

Response due to 6 γ lysine (tube 6) $= 27.5 - 23.0 = 4.5$

Lysine content of urine $= 6 (14.0/4.5) = 18.7$ γ/0.4 ml. $= 47$ γ/ml.

Calculations based on tubes 7, 8, and 9:

Response due to 0.6 ml. urine $= 24.0 - 4.5 = 19.5$

Response due to 6 γ lysine (tube 8) $= 28.5 - 24.0 = 4.5$

Response due to 6 γ lysine (tube 9) $= 32.5 - 28.5 = 4.0$

Average response due to 6 γ lysine $= (4.5 + 4.0)/2 = 4.25$

Lysine content of urine $= 6 (19.5/4.25) = 27.5$ γ/0.6 ml. $= 46$ γ/ml.

[a] Distilled $H_2O = 0$; complete opacity $= 100$.

A check on the linearity of the response is, of course, obtained when two levels of the assayed substance are added to the sample (*e.g.*, tubes 2 and 3, 5 and 6, and, 8 and 9 in Table I). We have also followed the general practice of always incorporating a conventional standard curve

in all assays. This gives one an additional check on the linearity of the response. Occasionally, there is observed a slight deviation from linearity at the low response end of the standard curve. In such a case, the calculations outlined in Table I are still valid providing the linear portion of the curve is extrapolated back, and an extrapolated "blank" value is used in the calculations rather than the measured blank.

A conventional standard response curve was included in the assay reported in Table I. Comparing the growth response obtained from 0.2 ml., 0.4 ml., and 0.6 ml. urine (tubes 1, 4, and 7) with this standard curve, values of 40, 31, and 28 γ/ml., respectively, were calculated. These values compare with the higher and much more concordant values of 46, 47, and 46 γ/ml. obtained with the improved procedure.

In addition to the necessary conditions already enumerated, there are certain inherent disadvantages in this method. The growth response measured for the largest sample plus standard amount of substance assayed (e.g., tube No. 9 in Table I) must not exceed the range of linear response, and is therefore smaller by a factor of 2 or 3 than that which can be obtained in the conventional procedure of reading assay values off a standard response curve. Furthermore, the growth response to a given sample aliquot, and the standard growth response, are obtained as the difference between 2 experimentally determined growth responses, and the experimental error is accordingly greater than when the response to the sample aliquot is compared to a standard curve based on a number of growth response determinations at different concentrations of the substance being assayed.

These disadvantages, however, are of a statistical nature and can be overcome by averaging the results of a sufficiently large number of determinations. The important consideration is that, by use of this method, consistent assay values have been obtained for samples which could never have been satisfactorily assayed by conventional procedures.

SUMMARY

A method is described by which reliable microbiological assays can be obtained on natural extracts containing relatively small amounts of the substance being assayed, in the presence of material which is toxic to the test organism employed.

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Estimation and Purification of Fumarase [1]

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INTRODUCTION

In 1941, Laki and Laki (1) reported the isolation from beef heart of a crystalline protein with properties of the enzyme fumarase. In repeating his methods, we have obtained evidence that the crystalline material is not, in fact, the enzyme.

No rational method for the estimation of fumarase activity has as yet been described. Laki and Laki calculated activity as though the reaction were of the simple zero order type, while Jacobsohn and da Cruz (2) believed the reaction to be of the first order. In this communication, a method of estimation of fumarase activity is described based on kinetic evidence (3).

EXPERIMENTAL

Estimation of Fumarase Activity

The reaction mixture was composed of the following: 10 ml. of solution containing enzyme, 0.1 M sodium fumarate or *l*-malate and phosphate buffer (pH 7.29, ionic strength 0.2). After 5–20 mins. at 30°C. in a water bath, 1 ml. of this solution was withdrawn, added to 0.5 ml. concentrated hydrochloric acid in 10 ml. water, and the unsaturation of the fumarate titrated with 0.02 M potassium permanganate. The endpoint was taken as that volume of permanganate when one drop (0.03 ml.) gave a pink color which persisted for 60 seconds. The molar fumarate concentration was calculated. Protein nitrogen was considered as that nitrogen insoluble in 5% trichloroacetic acid. It was determined ty the Hengar-Kjeldahl method.

Fumarase purity was calculated as total activity/total protein N (in g.) of the sample. Total activity was calculated in terms of initial rate in mM/second from the following:

$$\text{Total Activity} = \frac{V \times I.R.}{v},$$

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where V and v are total and sample volumes, respectively, and $I.R.$ is the initial rate of conversion of fumarate by the sample at pH 7.29 and 30°C. The initial rate can be calculated directly from one determination of the fumarate concentration as follows:

The conversion of fumarate to l-malate has been shown (3) to follow the kinetic equation

$$\alpha(x_0 - x) + \beta \ln\left(\frac{x_0 - x_e}{x - x_e}\right) = E \cdot t \tag{1}$$

(where x_0, x_e, and x are molar fumarate concentrations at the start, at equilibrium, and at time t (in sec.), respectively; E is enzyme concentration; and α and β are constants). From this equation, the initial rate of decrease of fumarase is

$$I.R. = -\left(\frac{dx}{dt}\right)_{x=x_0} = \frac{E(x_0 - x_e)}{\alpha(x_0 - x_e) + \beta}. \tag{2}$$

Substituting for E in Eq. (2) the appropriate value in Eq. (1), the initial rate becomes:

$$I.R. = \frac{x_0 - x_e}{[\alpha(x_0 - x_e) + \beta]t}\left[\alpha(x_0 - x) + \beta \ln\left(\frac{x_0 - x_e}{x - x_e}\right)\right]. \tag{3}$$

To convert initial rate from mole concentration change/sec. to mM/sec., the value of $I.R.$ from Eq. (3) was multiplied by 10.

The values of α and β at pH 7.29 and 30°C. were determined experimentally as 7900 and 780. Consequently, the appropriate equation for calculating initial rate in mM/sec. from one fumarate determination, x (in mole concentration) at time t was

$$I.R. = 1/t\left[4.4\ (0.1 - x) + 1.0 \log \frac{.0785}{x - .0215}\right]$$

since $x_e = .0215$ and $x_0 = 0.1000$.

Purification of Fumarase

Since there are some typographical errors in the procedure of Laki and Laki (1), and some modifications have been made, the purification will be described in full. Twenty-five pounds of beef heart were ground in a meat grinder and washed for 30 mins. with 15 gals. of ice water. Excess water was removed in a basket centrifuge, and the ground tissue heated with 8 l. of 0.01 M phosphate buffer (pH 6.7) to 45°C. The mixture was filtered through cheesecloth, the liquid was brought to pH 5.7, and chilled to 4°C. Twice the quantity of calcium phosphate gel described by Keilin and Hartree (4) was added to the cold solution, shaken, and removed by centrifuging.[2] The enzyme was eluted from the gel at 40°C. with the following volumes of 0.1 M phosphate buffer (pH 7.3): 1000, 1000, 500, and 500 ml. in succession. After cooling, 276 g./l. ammonium sulfate were added and the precipitate filtered off at 4°C. and discarded. An additional 109 g./l. (volume before salt addition) ammonium sulfate was added, and the precipitate collected in a Sharples supercentrifuge. It was dissolved in 200 ml. 0.1 M phosphate buffer (pH 6.7), cooled, and enough ammonium sulfate added to give a specific gravity of 1.152 at 4°C. The precipitate was filtered off at 0°C. and discarded.

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[2] Davydova (5) reports the use of alumina gel as a better adsorbent for fumarase than calcium phosphate gel.

Eleven g. of ammonium sulfate were added and the precipitate filtered off at 0°C. and dissolved in 20 ml. water. On cautious addition of ammonium sulfate, the crystalline protein of Laki and Laki was obtained from this solution. A salt fractionation of this solution gave samples of varying activity as described below.

RESULTS

A comparison of the purification process as described by Laki and Laki and as performed in our laboratory is shown in Table I. The

TABLE I

Purification of Fumarase (Procedure of Laki and Laki)

Stage of purification	Laki (1)	Author	
	Activity[a]	Recovery	Activity[a]
		Per cent	
A Heart extract	0.5	100	0.9
B Eluate	1.5	67	1.0
C 1st $(NH_4)_2SO_4$ precipitation		50	5.0
D 2nd $(NH_4)_2SO_4$ precipitation	13.0	28	8.5
E Crystals	14.5	6	6.1
F Crystals recrystallized	14.5	1	2.8
G Amorphous fraction		12	13.5

[a] Millimoles/sec./g. protein N.

purification achieved is quite good. However, in the crystallizing process, we repeatedly obtained a greater purity of the enzyme in the supernatant than was found in the crystals. Furthermore, the crystals lost activity greatly on recrystallization, and this activity was recoverable in the supernatant. Results of a second experiment are shown in Table II, where it is seen that salt fractionation was useful in further purification of the enzyme.

DISCUSSION

The most logical way of expressing enzyme purity is as per cent purity, but this is not possible until the pure enzyme has been prepared. Failing this, the next best method is to calculate potency, which is rate of reaction per unit dry weight of enzyme preparation. In first order reactions, potency can be expressed as the first order constant divided by weight of enzyme; in zero order reactions, as the initial rate

TABLE II

Further Purification of Fumarase

	Activity[b]	Recovery Per cent
Crystallization of D [a]		100
Crystals	2.4	28
Supernatant	3.2	51
Recrystallized once	1.0	4
Supernatant	2.1	22
Recrystallized twice	0.2	1
Supernatant	0.5	3
Salt fractionation of D [a]		100
D_1 1st fraction (crystals)	2.4	28
D_2 2nd fraction (amorphous)	4.5	47
D_3 3rd fraction	0.3	2
D_2 refractionated		
D_{2a} 1st fraction	2.7	5
D_{2b} 2nd fraction	5.7	34
D_{2c} 3rd fraction	3.0	4
D_{2b} refractionated		
1st fraction	4.5	13
2nd fraction	12.3	17
3rd fraction	3.0	1

[a] Actually, a preparation from a different experiment but corresponding to D in Table I.

[b] Millimoles/sec./g. protein N.

divided by weight of enzyme. The fumarase reaction, at least at the start, is of the zero order, and consequently the latter method was chosen. The process of calculation of the initial rate was resorted to since, by the graphical method, the initial rate is hard to estimate in an equilibrium process because the net rate of reaction is so soon slowed up by the reverse reaction. The units (mM/sec./g. protein N) were chosen for convenience.

Three explanations of the low activity of the crystals as opposed to the high activity of the supernatant appeared possible: (1) The crystals were progressively being inactivated during crystallization; (2) in the crystallization process, the crystals were separated from a coenzyme which remained in the supernatant; and (3) the crystals were not the enzyme.

The first explanation appeared improbable because usually 90% of the activity was recoverable in either supernatant or crystals. The

second did not appear correct since it also should be accompanied by a large net decrease in activity. Furthermore, no evidence of any coenzyme could be obtained, since neither cysteine, glutathione (both of which were reported as coenzymes (6)), nor boiled enzyme, had any activating effect on crystals of low activity, dialyzed enzyme, or acidprecipitated (6) enzyme. The results are consistent with the conclusion that the crystals are not the enzyme. The relatively high activity of the crystals was then due to their being formed in a flocculent mass in a solution of concentrated enzyme.

The procedure of Laki effects a 30-fold purification of the enzyme as obtained in an extract. In attempting further purification, the following should be considered: One pound of beef heart contains approximately $\frac{1}{8}$ g. of enzyme as here purified. Assuming an 80% loss in purification, 200 pounds of heart would then be required to give a reasonable quantity (5 g.) of enzyme for further purification.

Summary

A rational method of estimation of fumarase activity based on kinetic studies is described. Fumarase is not identical with the crystalline protein of Laki and Laki, and no evidence of a coenzyme could be obtained.

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Galactokinase

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In a previous paper (1) the name galactokinase was proposed for an enzyme which catalyzes the transphosphorylation between adenosine-triphosphate (ATP) and galactose. The existence of such an enzyme has been postulated by Kosterlitz (2), but no experimental evidence was given.

The reaction catalyzed by galactokinase is similar to the phosphorylation of other hexoses by hexokinase. The latter enzyme from yeast has been crystallized by Berger *et al.* (3), and Kunitz and McDonald (4), and shown to act on fructose, glucose and mannose, but not on galactose. It seemed important to decide whether organisms which utilize galactose possessed an entirely different enzyme or a hexokinase with a less stringent specificity.

Previous studies (1) showed that galactokinase activity in extracts of a lactose-fermenting yeast, *Saccharomyces fragilis*, was lost more easily than that of hexokinase on treatment with alcohol or acetone. A further proof of the difference between the two enzymes is now offered by the fact that partially purified extracts are richer in galactokinase than hexokinase, whereas the reverse is true for the crude extracts.

Another important point which had to be studied was that of the reaction product. In the hexokinase reaction the 6-phosphate is formed, but in the case of galactose this did not appear likely, since Grant (5) found that galactose-6-phosphate is not fermented by "galac" yeast. During the fermentation of galactose the only esters which he could detect were: fructose-1,6-diphosphate, glucose-6-phosphate and trehalose monophosphate. The only galactose ester isolated from natural sources has been galactose-1-phosphate which was obtained by Kosterlitz (6) from the liver of rabbits fed galactose. He showed also that this ester is fermented by "galac" yeast extracts.

The present study on galactokinase has shown that the reaction product is the Kosterlitz ester as follows:

$$ATP + galactose \rightarrow ADP + galactose\text{-}1\text{-}phosphate.$$
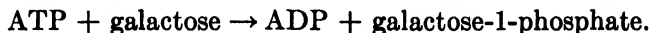
ESTIMATION OF THE ENZYME

The manometric (7) and titrimetric (4) methods which have been described for hexokinase can be used for galactokinase by substituting galactose for glucose. Measurements cannot be made by estimating the disappearance of easily hydrolyzable phosphorus because one labile phosphate of ATP is transformed into galactose-1-phosphate which is also acid-labile. Most experiments were made by the reduction method. This procedure is useful for serial work and might be easily adapted to much smaller amounts of materials simply by reducing the amount of reagents. The disappearance of free sugar is measured after precipitating proteins, hexose phosphate and adenosinephosphate with zinc sulphate and barium hydroxide (8). A similar procedure has also been used by Colowick et al. (9) as a check on manometric estimations.

The curve reproduced in Fig. 1 was obtained by incubation of the following mixture at 30°C. for 10 min.:

7.5 μM ATP, 2 μM galactose, 0.2 ml. of 0.1 M MgSO$_4$, 0.2 ml. of maleate buffer (10) of pH 6.0, and variable amounts of enzyme solution. Total volume 1 ml.

The reaction was started by adding the enzyme and stopped by addition of 0.5 ml. of 5% ZnSO$_4$ followed by 0.5 ml. of saturated barium hydroxide.

The same mixture without ATP was incubated as control, the ATP being added after the addition of the ZnSO$_4$ solution. Experience showed that no galactose disappears from this control.

After centrifuging, 1 ml. aliquots were analyzed as described by Somogyi (8) with Nelson's (11) arsenomolybdate reagent. A Klett-Summerson photocolorimeter with filter 52 was used. The micromoles of galactose which disappeared were calculated from the extinction of the experimental (E_E) and control (E_C) tubes as follows:

$$\Delta\mu M \text{ galactose} = 2(1 - E_E/E_C).$$

As shown in Fig. 1, the amount of galactose which disappears is a linear function of the enzyme concentration up to 1.3 μM.

The titrimetric method of Kunitz and McDonald (4) was used in some experiments. using galactose instead of glucose and reducing the amount of reagents to one-half, It was found that, within the limits of 0.1–0.5 ml. of 0.01 N NaOH, the acid formation is directly proportional to the amount of enzyme.

ENZYME PREPARATION

The enzyme can be obtained from galactose-adapted brewers' yeast but extracts of S. fragilis grown on whey as previously described (1) were found to be more active and were used in these studies. The cells were dried in thin layers at room temperature in about 15 hours and thereafter kept in a desiccator until used.

Extracts were prepared by suspending the dried yeast in 10 volumes of water, adjusting the pH to 6.4 with dilute ammonia, keeping it one hour at room temperature and then 20–24 hours in the ice-box. By centrifugation and avoiding increases in temperature an extract was obtained, 1 ml. of which catalysed the esterification of 30–70 μM of galactose in 10 min. under the conditions described. The hexokinase activity on glucose was usually 2–4 times greater.
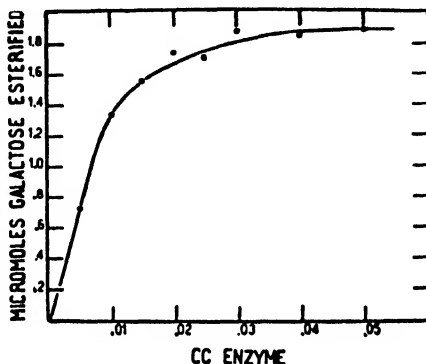
FIG. 1. Relationship between enzyme concentration and amount of galactose esterified. Measurements by copper reduction method. Details in text.

Fractionation with cold alcohol, acetone, or dioxane was tried, and it was found that galactokinase is rapidly destroyed. The best preparations were obtained by treatment with bentonite followed by ammonium sulfate fractionation.

When the crude extracts were treated with suitable amounts of bentonite (3–5 g./ 100 ml. of extract), most of the galactokinase remained in solution while about half of the inactive protein and nearly all the hexokinase was removed (Table I). The correct

TABLE I

Change in Galactokinase and Hexokinase by Purification

Activity estimated by the copper reduction method with 0.2 ml. enzyme solution. Purification as described in text.

	$\Delta\mu M$ Galactose	$\Delta\mu M$ Glucose
Crude extract	0.23 (0.50)	0.95 (1.84)
Purified extract	1.40 (0.40[a])	0.28 (0[a])

Numbers in brackets refer to a second experiment.
[a] Extract diluted 1/10.

amount of bentonite was ascertained by small scale trials in which galactokinase, hexokinase and total protein were estimated after treatment with different amounts of the adsorbent at pH 6.4.

The supernatant obtained after the treatment with bentonite was then mixed with one volume of saturated ammonium sulfate at room temperature. The precipitate

was discarded and to the supernatant 0.25 vol. of saturated ammonium sulfate was added. The precipitate was then dissolved in 1/20 of the original volume of water.

The solution obtained in this manner was usually 5–6 times more active than the crude extract. The ratio: activity/total protein was increased about 4-fold and the recovery was 30%. This extract proved to be suitable for the studies of the reaction product, and purification was not pursued further due to the labor involved in the preparation of the starting material.

PREPARATION

ATP was prepared according to Needham (12), and galacto-6-phosphate by the method of Levene and Raymond (13).

pH OPTIMUM

Maleate buffer (10) proved to be convenient because its buffering range covers the pH optimum of galactokinase and because it does not interfere with the activity of the enzyme or with the analytical procedure. It was ascertained that
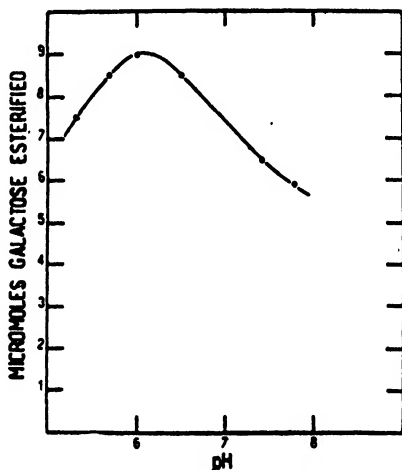
FIG. 2. Optimum pH of galactokinase. Estimations by copper reduction method with 0.008 M Mg and 0.2 ml. 0.1 M maleate buffer.

with the amount of buffer used the pH remained unchanged during the reaction. As shown in Fig. 2, galactokinase shows the greatest activity at pH 6 and is not affected to a great extent by changes from pH 5.3 to 7.8.

THE ACTION OF MAGNESIUM AND MANGANESE IONS

For these studies 1 ml. of the partially purified enzyme were dialyzed against 50 ml. of distilled water during 24 hr. at 5°C. A more prolonged dialysis led to complete inactivation.

Results are shown in Fig. 3. Both magnesium and manganese ions increase the activity of the enzyme. With magnesium the optimum concentration is 0.005 M, and with manganese 0.001 M. A greater activity is obtained with magnesium.
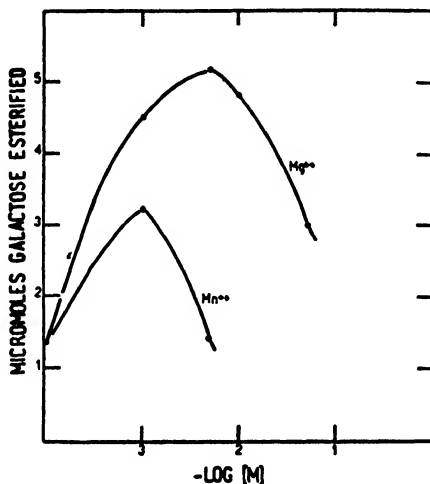
FIG. 3. Influence of magnesium (upper curve) and manganese (lower curve) ions on the activity of galactokinase. Estimations by copper reduction method. Maleate buffer of pH 7.
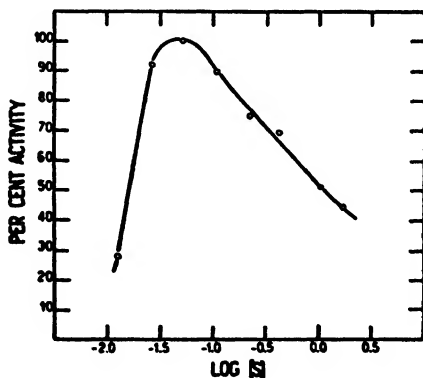
FIG. 4. Influence of galactose concentration on the activity of galactokinase. Titrimetric method; 5×10^{-3} M ATP; 30 min. at 5°C.

INFLUENCE OF GALACTOSE CONCENTRATION

Activity was measured by the titrimetric method at 5°C. The enzyme was a sample purified 4-fold by ammonium sulfate fractionation. Galactose was freed from traces of glucose by treatment with yeast as described by Stephenson and

Yudkin (14). The influence of changes in galactose concentration on the velocity of the reaction are shown in Fig. 4. Under those conditions, maximal rate was reached at about $3 \times 10^{-2} M$ and slowly decreased at higher concentrations. Care was taken that at low substrate concentration the reaction was not more than 75% completed at the moment the titration was carried out.

INFLUENCE OF ATP CONCENTRATION

Fig. 5 shows the results of changing the ATP concentration. Maximum activity was obtained at $1 \times 10^{-2} M$ and slowly decreased at higher concentration.
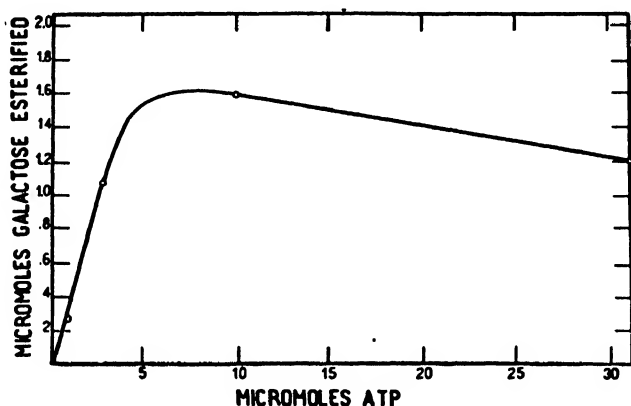
FIG. 5. Influence of ATP concentration on the activity of galactokinase. Copper reduction method; 15 min. at 5°C.; $2 \times 10^{-3} M$ galactose.

QUANTITATIVE ASPECTS OF THE PHOSPHATE TRANSFER

Colowick and Kalckar (7, 15) found that only one of the phosphate groups of ATP is transferred to glucose under the influence of hexokinase. When myokinase was added, two of the phosphate groups could be transferred because the following reaction was added to that of hexokinase: 2 ADP $\rightleftarrows$ ATP + AMP. In their study of myokinase its presence in yeast is not mentioned. However, an effect which may be attributed to a yeast myokinase is described by Kunitz and McDonald (4). They found that with crude hexokinase two acid equivalents per mol of ATP were liberated, whereas only one acid equivalent was liberated with the purified enzyme. Similar results are obtained with galactokinase but it has not been ascertained whether the effect is due to a yeast myokinase or to similar enzymes such as those described by Szent-Györgyi (16). The end-point of the reaction was studied in extracts of S. fragilis by both the titrimetric and copper reduction method.

Results obtained with the titrimetric method are shown in Table II. Two acid equivalents per mol ATP were liberated when crude extracts were used and the same result was obtained after ammonium sulfate fractionation. After treatment with suitable amounts of bentonite only one acid equivalent was liberated. The possibility

TABLE II

Acid Formation

Activity measured by titrimetric method at 5°C. with glucose and galactose. Final acid equivalents per mol ATP measured after reaction had been allowed to proceed to the end by incubation at 30°C. for one hour. Enzyme obtained by ammonium sulfate fractionation and dialysis.

	Final acid equivalents per mol ATP	
	Glucose	Galactose
Before bentonite	2.00	1.72
After bentonite	1.04	0.98

that a false end-point had been reached by inactivation of the enzyme was excluded by the fact that further addition of ATP produced another acid equivalent.

Results obtained by the copper reduction method were quite clear with the crude extracts. Two mols of galactose disappeared per mol ATP added. With purified extracts, the reaction usually stopped when about 0.8 mols of galactose disappeared per mol of ATP added. This was found to be due to inactivation of the enzyme because addition of more ATP did not produce any change. Presumably the cause of this inactivation was that, with the small amounts of substrate which had to be used, the reaction became so slow that enzymatic activity was lost before the end of the reaction.

THE NATURE OF THE HEXOSE ESTER

Preliminary experiments showed that the interaction of ATP and galactose with crude extracts led to the formation of an acid-stable fermentable ester. With partially purified extracts and by reducing the time of reaction, an acid-labile phosphate ester is formed.

The procedure of an experiment in which the labile ester was studied was as follows:

To a mixture of 150 μM of galactose and 140 μM of ATP in 10 ml. of 0.03 M magnesium sulphate, 0.5 ml. of enzyme purified with bentonite was added. After incubating 20 min. at 30°C. the reaction was stopped by adding excess mercuric acetate. The precipitate, which contained the proteins and adenosinephosphate, was washed and discarded. The supernatant plus the wash water was treated with hydrogen sulphide to remove the excess mercury.

The solution of the ester was then analyzed for inorganic, easily hydrolyzable (2 min. in 0.1 N H_2SO_4 at 100°C.) and total phosphate.

Reducing power was measured before and after hydrolysis and after fermentation with bakers' yeast. Results are shown in Table III.

Most of the ester (77%) is easily hydrolyzed. According to Kosterlitz (17), the synthetic galactose-1-phosphate is 100% hyrdolyzed in 2

min. in 0.1 N acid at 100°C. This ester is more acid-labile than glucose-1-phosphate. The phosphorus split under these conditions (112.5 μM) was accounted for by an increase in reduction (118.5 μM in terms of galactose).

TABLE III

Analysis of the Reaction Product of the Galactokinase Reaction

See text for details. Reducing power is expressed in μM of galactose.

Copper reduction		Phosphate	
	μM		μM
Direct	23.75	Inorganic	112.2
After hydrolysis minus direct	118.5	Acid-labile	112.5
After hydrolysis and fermentation minus direct	119.2	Acid-stable	33.0

The experiment shows that most of the ester is non-reducing, easily hydrolyzed by acid, and that the reducing portion liberated by acid is non-fermentable.

Controls were run at the same time to make sure that the amount of yeast used was sufficient to ferment totally a comparable amount of glucose and did not ferment added galactose.

The rest of the ester(s) formed (23%), which is not galactose-1-phosphate, is presumably a product of the action of other enzymes which transform the galactose-1-phosphate.

The same extracts were tested on galactose-6-phosphate and it was found that its reducing power remained unchanged, and that the bound phosphorus remained acid-stable. This confirms the finding of Grant (5) that galactose-6-phosphate is unattacked by "galac" yeast extracts.

DISCUSSION

Hexokinase appears to be the first enzyme involved in glucose utilization by most organisms, and presumably galactokinase has the same role in those which utilize galactose. Such is the case in the lactose-fermenting yeast studied in this paper and in galactose-adapted yeasts. In mammalian liver the enzyme is not so easily detected and probably accounts for the galactose-1-phosphate which was found by Kosterlitz (6) to accumulate after the feeding of galactose. Some studies carried out by Dr. M. Bacila in this laboratory, show that liver contains galactokinase and that it may be possible to study it in more detail.

That the reaction product is galactose-1-phosphate is indicated by the fact that the substance formed is non-reducing, that the phosphate is easily hydrolyzed with acid with appearance of reducing power, and that this reducing substance is not fermented by bakers' yeast. A still more convincing evidence will be at hand when this substance is compared with synthetic galactose-1-phosphate in relation to the enzymes which transform it further. These enzymes which, according to some preliminary work, lead to glucose-6-phosphate are now being studied.

It appears that the change from galactose to glucose takes place after phosphorylation at carbon atom one and it is tempting to postulate the formation of a 1,4-monophosphate with simultaneous inversion at C_4, thus leading to glucose-1,4-monophosphate which would finally lead to glucose-6-phosphate.

SUMMARY

An enzyme from a lactose fermenting yeast, *Saccharomyces fragilis*, which catalyzes the reaction of adenosinephosphate and galactose has been studied. With partially purified extracts, only one acid equivalent per mol of ATP is liberated in the reaction. The properties of the phosphate ester formed agree with those of synthetic galactose-1-phosphate in being: non-reducing, acid-labile, and non-fermentable by bakers' yeast after acid hydrolysis.

The reaction is therefore:

Adenosinetriphosphate+galactose→
adenosinediphosphate+galactose-1-phosphate.
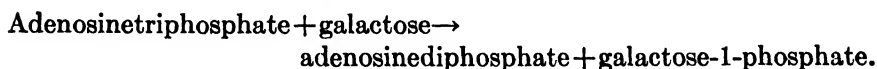
Maximum activity of galactokinase was obtained at pH 6 with 0.005 M magnesium or 0.001 M manganese, 3×10^{-2} M galactose, and 1×10^{-2} M ATP. The enzyme can also be detected in galactose-adapted brewers' yeast.

A method of estimation by copper reduction is described.

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The Reactivation of Raffinase

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INTRODUCTION

Herriott (1) had shown that yeast invertase inactivated by acid can be reactivated by following Anson and Mirsky's (2) procedure of allowing a solution of the inactivated enzyme to stand for varying intervals of time at pH 6–7. In previous investigations from this laboratory the influence of some 16 proteins (3) and 15 amino acids (4) on the reactivation of yeast invertase had been described. It became of interest to attempt the reactivation of raffinase, and if so, to see whether an antagonistic effect of cysteine and cystine could be obtained. This paper deals with the results of these studies.

EXPERIMENTAL

The enzymatic action was followed polarimetrically. Measurements of pH were made with a Leeds-Northrop glass-electrode type pH meter. The thermostat was maintained at a temperature of $35 \pm 0.05°C$. Various enzyme preparations extracted from yeast (*Saccharomyces cerevisiae*) were used; several were obtained from the Wallerstein Laboratories and Pfanstiehl Corporation. Other experimental details are similar to those described by Herriott (1) with minor modifications.

Reactivation Experiments

The various enzyme solutions were diluted to give an initial velocity of raffinase hydrolysis around 0.15–0.2°/min. When each enzyme preparation was brought to a pH below 1.0 and kept at this pH for 30 mins., followed by addition of alkali to a pH of 4.5 and allowing the mixture to stand for 30 mins., the enzyme was found to be completely inactive. However, when an acid-inactivated raffinase solution was brought to pH 6–7 and allowed to stand, significant reactivation occurred.

A typical experiment follows:

(A) Inactivation at pH less than 1.0. To 25 cc. of properly diluted raffinase solution dissolved in 0.04 M phosphate–citrate buffer (pH 4.5) was added 25 cc. of 0.369 N HCl.

The pH was usually less than 1.0. This was placed in a thermostat for 0.5 hr. at 35°C.

(B) Control for reactivation. To 25 cc. of the acid-enzyme mixture were added 25 cc. of 0.175 N NaOH to give a pH of 4.5.

(C) Reactivation at pH 6.1. To 25 cc. of the acid-enzyme mixture was added 25 cc. of 0.181 N NaOH to give a pH of 6.1. The 25 cc. of the enzyme mixture were then added to 150 cc. of 20% raffinose-hydrate solution in 0.03 M buffer at pH 4.5 and the velocity was determined.

TABLE I

Reactivation at pH 6.11

Reactivation for 30 mins. at pH 6.29 after inactivation at pH 0.98 for 30 minutes. pH of hydrolysis was 4.60.

Time in mins.	Δ Rotation in degrees	Rate in deg./min.
10	1.217	.0609
30	1.783	.0594
40	2.383	.0596
50	3.100 ·	.0620
	Mean rate	.0605

Reactivation at pH 4.60

Reactivation for 30 mins. at pH 4.60 after inactivation at pH 0.94 for 30 mins. pH of hydrolysis was 4.61.

Time in mins.	Δ Rotation in deg.	Rate in deg./min.
10	0.00	0.00
30	0.00	0.00
40	0.00	0.00
50	0.01	0.00
	Mean rate	0.00

This experiment was repeated with several other enzyme preparations under similar conditions, and the same results were obtained, which are like those obtained by Herriott (1) for invertase.

Optimum pH of Hydrolysis of the Reactivated Enzyme

It was now decided to determine the optimum pH of reactivation. Using a dried preparation containing raffinase (Wallerstein Laboratories Enzyme Scales) and keeping all other conditions the same, but

varying the concentration of alkali added to the acid-inactivated raffinase, data were obtained which are summarized in Table II.

The optimum pH of hydrolysis of the reactivated enzyme for this preparation is around 6.3, which is within the range obtained by Herriott (1) for invertase.

TABLE II

Effect of the pH of the Reactivating Medium on the Extent of Reactivation

pH of reactivating medium	Mean values of initial velocites in deg./min.	Per cent reactivation
4.6	0.000	0.0
5.5	0.047	25.0
6.1	0.060	31.9
6.5	0.063	33.6
7.0	0.046	24.5

Effect of Time of Reactivation on Extent of Reactivation

To make this determination, enzyme solutions containing Wallerstein Scales were inactivated and the conditions adjusted permitting reactivation. The reactivated enzyme solutions were allowed to stand at pH 6.3 for varying lengths of time. The information so obtained is recorded in Table III.

TABLE III

Effect of Time of Reactivation on Extent of Reactivation

The pH of reactivation was 0.93, while the pH of reactivation was 6.30, and the pH of hydrolysis was 4.6. The raffinase was inactivated for 0.5 hr. in each case.
Initial velocity 0.170°/min.

Time of reactivation in mins.	Rate in deg./min.	Per cent reactivation
0.5	0.002	1.2
1.0	0.015	8.8
15.0	0.046	27.1
30.0	0.050	29.5
90.0	0.055	32.4

The per cent reactivation obtained follows a similar trend to that found by Herriott for invertase (1).

Effect of Trypsin on Native Raffinase and Immediately Reactivated Raffinase

Previous experiments from this laboratory had shown that trypsin does not affect the activity of invertase at pH 6–7, but does prevent

the reactivation when added immediately after bringing to pII 6–7. It was decided to see whether raffinase behaves in a similar manner.

A raffinase preparation was inactivated for 0.5 hr. and then brought to pH 6.4. Immediately, 10 mg. of crystallized trypsin were added to the reactivating mixture and then allowed to stand for 0.5 hour in the thermostat. The control was run under the same conditions but contained no trypsin.

Rate in the presence of 10 mg. trypsin = 0.000°/min.
Rate of control   = 0.061°/min.

However, the addition of 10 mg. of trypsin to native raffinase when placed in the thermostat for 0.5 hr. resulted in the following rates:

pH 6.4, 10 mg. trypsin + raffinase: rate = 0.189°/min.
pH 6.4, raffinase: rate = 0.191°/min.

As with invertase (3), trypsin prevents the reactivation of raffinase when added immediately after bringing the pII to the reactivating pII, but does not affect native raffinase.

The Effect of Cysteine and Cystine on the Rate of Reactivated Raffinase

The reactivation process was now carried out in the presence of 20 mg. of cysteine hydrochloride and 20 mg. of L-cystine.

Results:

1. pH of reactivation was 6.6 in both cases.
   (a) hydrolysis rate of reactivated enzyme = 0.0543°/min.
   (b) hydrolysis rate of reactivated enzyme in presence of cystine
       = 0.0220°/min.
       Per cent decrease in extent of reactivation = 60%
2. (a) hydrolysis rate of reactivated enzyme = 0.0266°/min.
   (b) hydrolysis rate of reactivated enzyme in the presence of cysteine hydrochloride = 0.0597°/min.
       Per cent increase in extent of reactivation = 124%.

Thus an antagonistic effect of cysteine and cystine on the hydrolysis rate of reactivated raffinase was obtained, similar to that on invertase (4).

Bailey and co-workers (5) had shown that, at the same pH at which cysteine increased the rate of hog liver autolysis (pH 4), the autolysis in the presence of a quantity of KIO_3 sufficient to abolish the SH re-

action was considerably reduced. In view of these facts it was decided to try KIO₃ in the reactivating process.

Upon the addition of 20 mg. KIO₃ to an enzyme solution the hydrolysis rate of reactivated enzyme obtained was 0.000°C., while the control rate was 0.0663°/min., giving complete inhibition in the presence of KIO₃.

CONCLUSIONS AND SUMMARY

1. Experiments have shown that acid-inactivated raffinase may be reactivated.

2. The pH optimum of reactivation for the raffinase preparation used was around 6.3.

3. The per cent of reactivation of raffinase increases with the time of standing at the optimum pH.

4. Trypsin does not affect native raffinase, but does prevent activity when added immediately after conditions are adjusted permitting reactivation.

5. Cysteine increases the extent of reactivation of acid inactivated raffinase while cystine inhibits it.

6. Potassium iodate completely inhibits the reactivation of raffinase.

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The Effect of Succinylsulfathiazole on Pteroylglutamic Acid Deficiency during Lactation in the Rat [1]

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INTRODUCTION

The production of a pteroylglutamic acid deficiency in young rats by means of sulfonamide-containing diets and its cure and prevention by low levels of the synthetic vitamin are well known (1). Recently it has been shown that the stress of lactation in the adult rat will, in three weeks, produce this vitamin deficiency in the absence of a sulfonamide and that high levels of the vitamin are necessary for its prevention (2). The present report shows that the addition of succinylsulfathiazole to depress intestinal synthesis will further accentuate this deficiency induced by lactation.

EXPERIMENTAL

The short-term procedure for studying lactation is the same as previously described (2, 3). Adult stock animals of the Long-Evans strain, together with their litters, were placed on the experimental diets at parturition. The litters were limited to 6 young, preferably 3 males and 3 females. All animals, mother and young, were weighed every 5 days and the young were weaned on the 21st day following parturition. Diet was given *ad libitum* and the food intake was measured. Three to five series of litters (each series consists of 10–12 litters) have been used for each experimental group in this study.

The criteria used for lactation performance with this procedure are: (1) the weaning weight of the young; (2) the weight change of the lactating mothers during the 21-day

period; and (3) the maternal leucocyte count at the end of lactation. These criteria have been discussed previously (2, 3).

The basal purified diet 836 [2] supplemented with biotin[3] was used throughout this study. Synthetic pteroylglutamic acid (PGA) was incorporated in the diet at two levels, 275 and 550 γ/100 g. diet. Succinylsulfathiazole (SST) was used at a 1% level and replaced an equivalent amount of sucrose.

Total and differential white blood counts were carried out by standard procedures at the time of weaning (Day 21). Erythrocyte counts were carried out on many animals at the same time. Additional white blood counts were made on some PGA-deficient animals receiving the SST-containing diet near the beginning of lactation (Day 5) and 3 weeks following the end of lactation (Day 41–42).

RESULTS AND DISCUSSION

Table I shows that the addition of SST to the basal diet has markedly accentuated the PGA deficiency induced by the stress of lactation. On the basal diet lacking PGA, the mothers lose approximately 30 g. in body weight and wean young averaging 43 g. (females) to 44 g. (males). At weaning, the maternal leucocytes average 4755 cells of which 80%, or 3810, are lymphocytes and monocytes, and 20%, or 945, are granulocytes (PMN). The erythrocytes average 7.30 million (range 4.7–9.2).

In contrast, the mothers receiving the basal diet containing 1% SST lose twice as much weight during lactation, approximately 67 g., and wean young averaging only 33 g. (females) to 35 g. (males). At weaning the maternal leucocytes are markedly decreased, i.e., 2680 cells of which 94%, or 2500, are lymphocytes and monocytes, and only 6%, or 180, are granulocytes. In addition, the erythrocyte count has dropped to 5.75 million (range 3.2–7.8). The average daily food intake decreased from 26.3 to 21.3 g. The deficiency on this diet was so acute that almost 20% of the mothers placed on the diet at parturition died before the young were weaned at 21 days. Death of the mother during

[2] The basal diet 836 has been used for the previous studies on lactation (2, 3). It consists of 24% alcohol-extracted casein, 64% sucrose, 8% hydrogenated vegetable oil (Crisco or Primex) and 4% salts No. 4. Crystalline vitamins added/kg. diet are: 5 mg. 2-methyl-1,4-naphthoquinone, 5 mg. thiamine HCl, 5 mg. pyridoxine HCl, 10 mg. riboflavin, 10 mg. p-aminobenzoic acid, 20 mg. nicotinic acid, 50 mg. calcium pantothenate, 400 mg. inositol and 1 g. choline chloride. For this study 300–600 γ crystalline d-biotin was included/kg. diet. One cc. of a fat-soluble vitamin mixture containing 6 mg. α-tocopherol, 115 chick units vitamin D, 800 U.S.P. units vitamin A and 650 mg. corn oil (Mazola) was given weekly to each litter.

[3] The beneficial effects of biotin on lactation in the rat may be noted chiefly in the weaning weights of the young (4).

TABLE I

Effect of Succinylsulfathiazole (SST) on Pteroylglutamic Acid (PGA) Deficiency during Lactation

Supplement/100 g. diet	No. of litters	Av. weaning wt. young[a]	Wt. change of mother during lactation[b]	Av. daily food intake of mother	Maternal blood counts at weaning			
					RBC×10[6]	Total WBC/mm.[3]	Lymphocytes and monocytes	PMN
		g.	g.	g.				
Basal diet								
0	55	44 43	−30.3[55] (−66 to +15)	26.3[55]	7.30[31] (4.7–9.2)	4,755[51] (1,750–8,700)	3,810 (1,190–8,350) 80%	945 (0–2,775) 20%
275–550 γ PGA	57	50 48	+6.4[57] (−21 to +35)	31.9[57]	8.11[22] (7.1–10.2)	8,560[55] (4,500–16,500)	5,955 (2,550–13,135) 69%	2,605 (760–6,300) 31%
Basal diet + 1% SST								
0	33	35 33	−66.6[26] (−108 to −40)	21.3[26]	5.75[10] (3.2–7.8)	2,680[26] (1,240–6,480)	2,500 (1,240–6,480) 94%	180 (0–1,135) 6%
550 γ PGA	42	50 48	+10.3[41] (−28 to +37)	32.5[41]	8.39[18] (6.4–10.2)	8,145[38] (3,850–16,200)	5,835 (2,710–13,445) 71%	2,310 (525–5,680) 29%

[a] In this column the upper figure refers to the average weaning weight of male rats and the lower figure to the average weaning weight for female rats.

[b] Superscript numbers in parentheses in this table indicate the number of animals on which the average is based. Only mothers weaning 5–6 young are included in the columns on weight change, food intake, and maternal blood counts.

lactation has been an exceedingly rare occurrence in our experience, either on the basal diet without SST or on diets deficient in other B vitamins or in protein, despite an even greater loss in body weight. The percentage of young weaned by the surviving rats on the SST-containing diet was 87% in contrast to the 97% weaned on the diet without SST. It may be mentioned also that many of the surviving rats showed ulceration and "caking" of the mammary glands. This has been noticed previously by Vinson and Cerecedo (5) in lactating Long-Evans rats maintained since weaning on their basal diets lacking in PGA.

The marked accentuation of the blood dyscrasia by the addition of SST is shown by the fact that 96% of the white blood counts for rats maintained on this diet fall below the normal range for total leucocytes (i.e., 6000 cells) and 100% are below the normal minimum for granulocytes (i.e., 1250 PMN). Furthermore, 88% of the counts dropped to less than 500 granulocytes while 31% showed no granulocytes whatsoever. The corresponding percentages for the blood counts of lactating mothers maintained on the same diet without SST are: 75%, 69%, 41%, and 4%. On the SST-containing diet almost half (46%) of the rats show a slight to marked degree of anemia while less than 10% of the rats not receiving SST show any depression of the erythrocyte count (i.e., below 6 million/cm.³). The data in Table I show the importance of intestinal synthesis in furnishing part of the PGA requirement during lactation.

Since the maternal leucocytes for the lactating mothers on the SST-containing diet were so low at the end of lactation, it was considered advisable to check the blood counts at the beginning of lactation. Previous studies carried out with rats maintained on the basal diet without SST had shown that the blood counts were still normal on the 5th day of lactation and that the blood dyscrasia developed between the 5th and 21st days of lactation (6). Typical values for rats maintained on the SST-containing diet are given in Table II. The data show that the white blood counts were entirely normal at the beginning of lactation despite the extremely rapid development of the blood dyscrasia in 16 days which resulted in a drop from 10,145 total leucocytes to 3170 leucocytes and from 28%, or 2855, granulocytes to 5%, or 205, cells. The decrease in circulating lymphocytes and monocytes is also marked, namely, from 7290 to 2965 cells. It may be noted that during the first 5 days of lactation these rats increased slightly in body

TABLE II

*Changes in Maternal Leucocytes and Body Weight during Lactation for
Rats Maintained on the PGA-Deficient Diet containing SST*

Rat number	Day of lactation	Total WBC/mm.³	Lymphocytes and monocytes		PMN		Wt. change of mother from day 1 (parturition)
W7095	5	8,750	74%	6,475	26%	2,275	g. + 3
	21	7,200	90%	6,480	10%	720	−41
B6634	5	8,540	66%	5,635	34%	2,905	+ 4
	21	1,350	92%	1,240	8%	110	−72
B9811	5	15,100	74%	11,175	26%	3,925	+ 7
	21	3,500	97%	3,395	3%	105	−63
W7078	6	8,100	74%	5,995	26%	2,105	+ 1
	22	2,150	98%	2,105	2%	45	−55
B9810	5	10,250	70%	7,175	30%	3,075	+ 2
	21	1,650	97%	1,600	3%	50	−95
Averages (5 rats)	5	10,145	72%	7,290	28%	2,855	+ 3.4
	21	3,170	95%	2,965	5%	205	−65.2

weight (1–7 g.) so that the marked loss in body weight of 41–95 g. also occurred during the 16 days.

Previous studies by Nelson and Evans (3) have shown by a comparison between lactating and non lactating mothers maintained on the basal diet that the strain of lactation alone was responsible for the marked weight loss during lactation. For further confirmation, several severely affected animals were kept on the SST-containing diet for 3 weeks following the end of lactation. Table III shows that in these 3 weeks a greater part of the weight lost during the 21 days of lactation was regained and the blood dyscrasia was partially corrected. An average of 48 g. was gained in comparison to the previous loss during lactation of 68 g. At the same time the total leucocytes increased from 2120 to 6605 cells while the granulocytes jumped from 2% (50 cells) to 25% (1620 cells) even though SST was present in the diet. The data in Tables II and III emphasize the strain of lactation as exemplified by the rapid development of the blood dyscrasia during this period and

TABLE III

Changes in Maternal Leucocytes and Body Weight Following Lactation
for Rats Maintained on the PGA-Deficient Diet containing SST

Rat number	Day of lactation	Total WBC/mm.³	Lymphocytes and monocytes		PMN		Wt. change of mother from day 1 (day 21)
GH7911	21	2,850	96%	2,735	4%	115	— 56 *g.*
	41	8,900	82%	7,300	18%	1,600	— 14(+42)
W7409	21	1,200	100%	1,200	0%	0	— 79
	41	6,450	67%	4,320	33%	2,130	— 26(+53)
GH2911	21	1,650	100%	1,650	0%	0	— 48
	41	6,500	81%	5,265	19%	1,235	+ 1(+49)
W7805	21	2,500	94%	2,350	6%	150	— 66
	42	5,700	74%	4,220	26%	1,480	— 8(+58)
B9464	21	3,300	98%	3,235	2%	65	— 56
	41	9,150	72%	6,590	28%	2,560	— 12(+44)
B1554	21	1,800	100%	1,800	0%	0	— 65
	41	5,050	73%	3,685	27%	1,365	— 23(+42)
B1755	21	1,550	98%	1,520	2%	30	—105
	41	4,500	78%	3,510	22%	990	— 54(+51)
Averages (7 rats)	21	2,120	98%	2,070	2%	50	— 67.9
	41	6,605	75%	4,985	25%	1,620	— 19.6(+48.3)

the improvement in condition after lactation has ended despite the continued presence of SST in the diet.

Despite the accentuation of the deficiency by the sulfonamide, high levels of synthetic pteroylglutamic acid are as beneficial when SST is present as when it is absent from the diet (Table I). Only the one level (550 γ PGA/100 g. diet) was tested in the presence of SST but the data show that it is adequate, inasmuch as the weaning weights of the young, the weight gain of the mother, the erythrocyte count, and the total and differential white blood counts are practically identical, regardless of the presence or absence of SST. No additional deficiency has been brought out by the addition of SST although lactation on the purified diet supplemented with 10 crystalline B vitamins is still not equivalent

to that obtained by supplementation of the purified diet with liver eluate powder by the use of a good stock diet (2, 3) when judged by the weight gain of the mother and the maternal leucocytes at weaning. Apparently the unknown factor (or factors) still missing from the purified diet is not affected by the presence of SST and presumably is not furnished by intestinal synthesis in significant amounts (unless the microorganisms responsible are resistant to SST). Evidence that the rat needs additional unknown factors under certain conditions (vegetable protein diet or purified diets using heat-coagulated albumin or casein exhaustively extracted with alcohol) has recently been reported by Cary and Hartman (7), Zucker and Zucker (8), and Bowland *et al.* (9).

SUMMARY

The addition of succinylsulfathiazole to depress intestinal synthesis markedly accentuates the pteroylglutamic acid deficiency induced by the strain of lactation in the rat. The leucopenia and granulocytopenia produced under these conditions are especially severe. Furthermore, anemia occurred in many of the lactating mothers. Further confirmation of the role of lactation in inducing the pteroylglutamic acid deficiency is shown by the fact that maintenance on the identical diet for 3 weeks following the end of lactation results in improvement in body weight and partial correction of the blood dyscrasia even though succinylsulfathiazole is still present in the diet. Despite the accentuation of the deficiency, high levels of synthetic pteroylglutamic acid are as beneficial when succinylsulfathiazole is present as when it is absent from the diet.

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Catalase and Peroxidase in Germinating Cottonseed

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INTRODUCTION

As part of the research program on the storage and chemical treatment of seeds to inhibit or prevent heating and deterioration during storage (1, 2, 3, 4, 5), an investigation was undertaken of the activity of catalase and peroxidase in seeds and seedlings of cotton. Although they were among the earliest enzymes to be discovered (6) and despite the fact that both of them have been extensively investigated, little is known concerning their physiological role (7). To obtain information concerning the role these enzymes play in the production of energy, and especially in connection with the development of methods of inhibiting their activity in seeds, these enzymes were investigated during the period of change accompanying early germination.

Germination of seeds generally induces a change in their catalase and peroxidase activities. Willstätter and Pollinger (8) noted an increase in the peroxidase activity of extracts of wheat, rye, and oat seedlings during germination, the peroxidase content of wheat extracts having increased 10-fold in one week. Increases in both catalase and peroxidase activities during germination of the seeds of *Juniperus scopulorum* were reported by Afanasiev and Cress (9). Deleano (10) investigated the changes in catalase and peroxidase during germination of seeds of *Ricinus communis* and found that the catalase activity of extracts increased very rapidly at the beginning of germination, passed through a maximum, and diminished as rapidly as it increased at the beginning. Peroxidase activity, on the other hand, increased more slowly, but after 9 days of germination, when the catalase activity had passed its maximum, peroxidase activity was increasing and continued to increase for as along as 22 days. Bach *et al.* (11) found a similar relationship between these two enzymes during the germination of wheat seeds. Before germination, the ratio of peroxidase to catalase activity in extracts of the seed was approximately 2, after 3 days germination this ratio was 1, but on the fourth day the ratio had increased to 9.

Loew (12) reported that cottonseed contains considerable quantities of catalase, a fact which was confirmed by Olcott and Thornton (13) who stated that cottonseed is

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one of the best plant sources of this enzyme. The latter authors also reported that, during the first 48 hours of germination, the catalase activity of extracts of the seedlings increased to a value of 120–140% of the original. Although resting cottonseed contains little peroxidase, it has been reported by Altschul and Karon (14) that there is appreciable peroxidase activity in extracts of cotton seedlings.

One must distinguish between enzyme activity as manifested in tissue homogenates and extracts and that of enzyme content *in vivo*. For example, Morris *et al.* (15) state, "Reported increases in enzyme activity do not always mean a parallel increase in enzyme content." This is particularly true for the evaluation of extracts from germinating seeds because the cellular structure of the tissue is changing as the germination progresses. An increase in ease of extraction of an enzyme as a result of germination may be interpreted erroneously as representing an increase in enzyme content. Simultaneous analysis of germinating seeds for two or more enzymes may, however, simplify the problem of interpretation of *in vitro* observations

METHODS

Extraction of Enzymes

Cottonseed was moistened for ½ hour, placed on moistened filter paper, and allowed to germinate in the dark in shallow trays at 29°C. Unless otherwise indicated, no agent was used to sterilize the surfaces of the seeds prior to germination because, in most cases, the seeds had a high degree of viability even without prior surface sterilization. When the germination had progressed for the desired length of time the seedlings were decorticated, mixed with an excess of cold acetone, and macerated in a Waring Blendor for a few minutes. The resulting mixture was centrifuged, the extract discarded, and the residue re-extracted 3 times with acetone in the same manner. The fat-free powder was dried in an air-stream, reground to pass through a 60-mesh screen, and stored at −16°C. until used. This material is referred to as acetone-dried tissue. The same procedure was used in preparing extracts from ungerminated seeds.

Enzyme extracts were prepared by homogenizing 2-g. samples of the acetone-dried tissue with 15 ml. of $M/15$ phosphate buffer at pH of 7. An additional 5 ml. of buffer was added to the homogenate and the mixture allowed to autolyze at 5°C. for about 16 hours. Toluene was added to prevent bacterial growth and was effective at this temperature. The autolyzed mixture was centrifuged and the residue re-extracted 3 times with 10 ml. each time of the neutral phosphate buffer. The extracts were combined and diluted to a 50 ml. volume with neutral buffer. Approximately 90% of the extractable catalase and peroxidase is extracted by this procedure.

Determination of Enzyme Activity

Catalase determinations were made with a Warburg constant-volume manometric apparatus at 0°C. by measuring the rate of evolution of oxygen. The test mixture

consisted of 2 ml. of $M/15$ neutral phosphate buffer, 0.15 ml. of hydrogen peroxide solution (one part of commercial 30% hydrogen peroxide freshly diluted with 99 parts of water), and sufficient enzyme solution and water to make the final volume of the test solution equal to 3.3 ml. When the enzyme concentration was adjusted so that 25–55% of the hydrogen peroxide was destroyed in 10 minutes, the rate constant calculated for a first order reaction was found to be proportional to the catalase concentration. The catalase activity was, therefore, determined under conditions which resulted in a first order reaction and the activity expressed as a first order rate constant.[2]

Peroxidase activity was determined by the manometric method described by Altschul and Karon (14) and the results were also expressed in terms of a first order rate constant. The determinations were made at 30°C. in Warburg vessels having two side-arms. The reagents used in the test comprised 2 ml. of $M/15$ phosphate buffer at pH of 6.8, 0.15 ml. of a freshly prepared 3% solution of pyrogallol, 0.15 ml. of hydrogen peroxide solution (one part of commercial hydrogen peroxide freshly diluted with 99 parts of water), 0.30 ml. of a concentrated suspension of cottonseed meal (source of catalase), enzyme solution, and water to make a total volume of 3.3 ml.

To eliminate catalase activity in the extract before testing for peroxidase, the enzyme extracts were treated with pyrogallol according to the method of Kasanski (16) as modified by Altschul and Karon (14). This was accomplished by the addition of pyrogallol (1.25 g.) to 25 ml. of each enzyme extract, after which the mixture was incubated at 3°C. for 24 hours. Following incubation the solution was clarified by centrifugation and dialyzed against running distilled water for 24 hours at 3°C. The dialyzed solution was again clarified by centrifugation. This solution was devoid of catalase activity.

Kasanski (16) states that the pyrogallol treatment does not appreciably reduce the peroxidase activity of an enzyme extract. This observation was confirmed in the case of cottonseed peroxidase by estimating the activity of this enzyme in extracts prepared from resting seed and from 5-day old seedlings before and after treatment with pyrogallol. In the case of extracts prepared from resting seed, the original peroxidase activity was estimated to be 0.20 units/ml., and after treatment of the extract with pyrogallol and correction for change in volume it was found to be 0.16 units/ml. The extracts prepared from the cotton seedlings contained 0.59 units of peroxidase/ml. compared to 0.63 units/ml. after treatment with pyrogallol.

RESULTS

Changes during Germination

Seeds of the Delfos 651 variety of cottonseed, grown in Stoneville, Mississippi, and harvested in September, 1943, were investigated.

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[2] The value of the first order rate constant is calculated from the following equation:

$$K = \frac{2.303}{t} \log \frac{V_\infty}{V_\infty - V_t},$$

where V_∞ is the maximum volume of oxygen evolved when a large excess of catalase is used and V_t is the volume of oxygen evolved after a period of reaction of t minutes.

Analyses were made of samples of seeds before germination and at successive daily intervals during a 5-day germination period. Samples were ground, extracted with acetone, dried as previously described, and extracts were made of the acetone-dried tissues. One portion of each extract was analyzed for catalase activity and another portion was treated with pyrogallol, after which it was analyzed for peroxidase activity with the results given in Table I. In the course of the 5-day

TABLE I

Extractable Catalase and Peroxidase in Germinating Cottonseed

Length of ger-mination	Nitro-gen con-tent	Acetone-dried tissue			Enzyme extracts			
		Extractable enzyme[a] (activity/min.)[b]		Ratio, catalase to per-oxidase	Per cent of total nitrogen in		Purity of enzyme preparations (activity/min./mg. N)	
		Catalase	Peroxidase		Original[c] extract	Catalase-free[d] extract	Catalase	Peroxidase
Days	*Per cent*							
0	7.8	172	1.8	98 .	37.4	21.3	5.9	0.1
1	7.8	97.6	0.7	132	30.5	13.9	4.1	0.1
2	8.6	161	1.6	99	39.4	11.1	4.7	0.2
3	8.2	34.6	5.7	6.1	41.2	6.4	1.0	1.1
5	8.2	30.5	38	0.8	36.1	2.9	1.0	16.0

[a] Total amount of enzyme extracted from 1 g. of the acetone-dried tissue.
[b] Activity is expressed in terms of the first order rate constants.
[c] Refers to buffer extract of acetone-dried tissue.
[d] Refers to extract which has been treated with pyrogallol to destroy the catalase activity.

period of germination the amount of extractable catalase decreased to 18% of its value in the original seed, whereas the extractable peroxidase increased 21-fold in the same period.

In Table I there are also given the nitrogen contents of the acetone-dried tissues, enzyme extract, and the pyrogallol-treated catalase-free solution. It is evident from the data in this table that the nitrogen contents of the tissue increased slightly with increased time of germination, whereas the percentages of extractable nitrogen did not vary appreciably during germination. The amount of nitrogen remaining in the catalase-free solution following pyrogallol treatment decreased as

the age of the seedlings from which these solutions were prepared increased. Examination of the relationship between enzyme activity and nitrogen content of the extracts reveals that the total amount of extractable catalase not only decreased with the length of germination but the "purity" of the enzyme expressed in terms of catalase activity
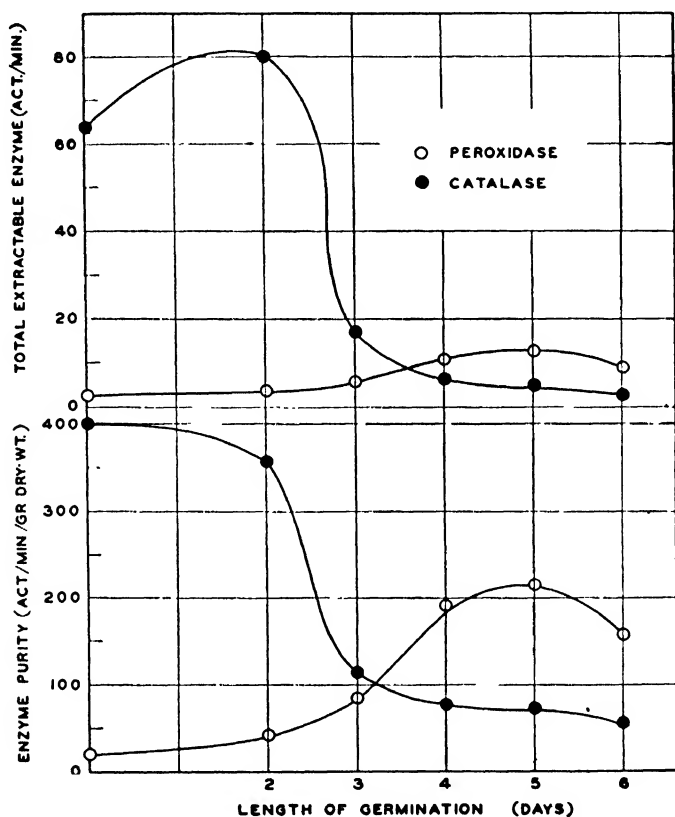
FIG. 1. Changes in extractable catalase and peroxidase in germinating Delfos 3506 variety cottonseed.

per unit of nitrogen in the extract also decreased. On the other hand, the "purity" of the peroxidase both in the original extract and in the catalase-free solution increased with increased time of germination and growth of the seedling. A similar relationship between catalase and peroxidase was observed in a series of experiments on Delfos 3506

variety of cottonseed harvested at Stoneville, Mississippi, during October, 1942, and stored for one year at room temperature prior to germination.

Distribution of Catalase and Peroxidase in Radicles and Hypocotyls and Cotyledons

In another series of experiments with Delfos 651 variety, cottonseed catalase was found to decrease and peroxidase to increase in both the radicles and the hypocotyls and cotyledons of the germinated seedlings. The radicles were separated from the hypocotyls and cotyledons prior to drying the seedlings with acetone and extracting the enzymes. Results of the analyses of the extracts are given in Table II. More

TABLE II

Distribution of Extractable Catalase and Peroxidase in the Radicle and the Hypocotyl and Cotyledons of Cotton Seedlings

Length of germina-tion	Portion of seed or seedling	Acetone-dried tissue			Enzyme extracts	
		Extractalbe enzyme[a] (activity/min.)[b]		Ratio, catalase to peroxi-dase	Purity of enzyme preparations (activ-ity/min./g. dry matter)	
		Catalase	Peroxi-dase		Catalase	Peroxi-dase
Days						
0	Entire seed kernel	180	0.7	253	1000	—
3	Hypocotyl and cotyledon	24	1.3	18	100	100
3	Radicle	5.5	1.0	5.6	400	200
5	Hypocotyl and cotyledon	5.0	2.9	1.7	100	500
5	Radicle	2.8	1.3	2.2	100	500

[a] Total amount of enzyme extracted from 1 g. of acetone-dried tissue.
[b] Activity is expressed in terms of the first order rate constants.

catalase and peroxidase is extractable from the hypocotyl and cotyledons than from the radicles, but the total amount of material extractable from the radicles is much less than that from the hypocotyls and cotyledons and the "purity" of the enzyme material from the former is greater than from the latter.

Distribution of Catalase and Peroxidase in Extract and Residue

In his investigations on the distribution of catalase in various plants, Loew (12) found that not all of the catalase was extractable by the methods he used. Almost 50% of the catalase in cottonseed remained in the residue after extraction. He designated the insoluble form as α-catalase and the soluble form as β-catalase. It is doubtful whether there were 2 forms of catalase or whether there is a sharp distinction between the extractable and non-extractable material. It is probable that some portions of the catalase are more strongly bound to the tissue than others, and that the amount of extractable catalase would depend both on the state of the tissue and the method used for its extraction. With any given method of extraction, however, any variation in the amount of extractable enzyme as a function of changes in the tissue may assume significance with respect to the mobility of the enzyme in the tissue.

To determine the effect of germination upon the extractability of catalase and peroxidase, both the extracts and residue were analyzed with the results given in Table III for seeds of the Delfos 651 variety

TABLE III

Effect of Germination of Cottonseed upon the Extractibility of Catalase and Peroxidase

Length of germination	Variety	Catalase[a] (activity/min.)[b]				Peroxidase[a] (activity/min.)[b]				Ratio total catalase to total peroxidase
		Extract	Residue	Total	Per cent extracted	Extract	Residue	Total	Per cent extracted	
Days										
0	Delfos 651	378	6	384	99	8.5	12.2	20.7	59	19
5	Delfos 651	50	310	360	14	38	16.8	54.8	69	7
0	D&PL 45	422	74	496	85	1.7	1.4	3.1	55	160
2	D&PL 45	250	380	630	40	2.2	1.4	3.6	61	175
3	D&PL 45	50	170	220	23	4.6	4.1	8.7	53	25
4	D&PL 45	2	68	70	3	1.0	1.4	2.3	38	30
5	D&PL 45	4	66	70	6	1.7	3.3	5.0	34	14
6	D&PL 45	6	125	131	5	4.4	7.6	12.0	37	11
7	D&PL 45	9	100	109	8	1.4	3.6	5.0	28	22

[a] Amount obtained from 1 g. of acetone-dried powder.
[b] Activity is expressed in terms of the first order rate constants.

before, and at the end of, a 5 day germination period. It is evident that, although an appreciable decrease in the amount of extractable catalase occurred during germination, the total amount of catalase (sum of activities in extract and residue) decreased only slightly. The

distribution of peroxidase between extract and residue was not appreciably affected by germination.

Somewhat similar results were obtained by germination of D&PL 45 variety of cottonseed harvested at Stoneville, Mississippi, during September, 1945. In this experiment, enzyme analyses were made on seedlings from the second through the seventh day with the results given in Table III.[3] The amount of extractable catalase decreased regularly during the first 4 days of germination and then remained at a low value for the remainder of the period. The total amount of catalase also decreased during germination in contrast to the results of the previous experiment where there was no such change, but the magnitude of the change in total catalase was far less than the change in extractable catalase. Whereas the percentage of the total catalase which could be extracted decreased from 85% for resting seed to 3% after 4 days germination, the change in percentage of extractable peroxidase during germination was much less and not as regular. The greatest amount of extractable peroxidase was found in the 2 day old seedlings and the least in the 7 day old seedlings. The ratio of total catalase activity to total peroxidase activity decreased during germination from a high of 175 on the second day to a low of 11 on the sixth day.

DISCUSSION

In tissue containing oxidase and catalase but no peroxidase, the steps and energy relationships for the reduction of oxygen to water are:

$$2\epsilon + 2H^+ + O_2 = H_2O_2; \Delta F = -31,470 \text{ cal.}$$
$$H_2O_2 = \tfrac{1}{2} O_2 + H_2O; \Delta F = -25,090 \text{ cal.}$$

$$2\epsilon + 2H^+ + \tfrac{1}{2} O_2 = H_2O; \Delta F = -56,560 \text{ cal.}$$
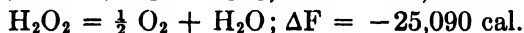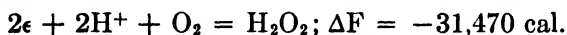
In tissue containing oxidase and peroxidase but no catalase, the steps and energy relationships for the same reaction are:

$$\epsilon + H^+ + \tfrac{1}{2} O_2 = \tfrac{1}{2}H_2O_2; \Delta F = -15,735 \text{ cal.}$$
$$\epsilon + \tfrac{1}{2}H_2O_2 + H^+ = H_2O; \Delta F = -40,825 \text{ cal.}$$

$$2\epsilon + 2H^+ + \tfrac{1}{2} O_2 = H_2O; \Delta F = -56,560 \text{ cal.}$$
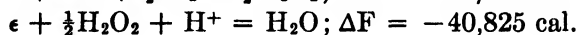
The total free energy released in the presence of either catalase or peroxidase is the same, but there are differences in the availability of the free energy. In the peroxidase-directed reaction, all of the free energy is coupled to oxidation-reduction reactions and is thus potentially available for functions requiring work. In the catalase-directed reaction, however, 44% of the total free energy released is not neces-

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[3] The seeds were sterilized by soaking for 15 minutes in a 0.1% solution of mercuric chloride prior to germination.

sarily coupled to an oxidation-reduction reaction and may be dissipated as heat.[4]

It might be assumed, therefore, that rapidly growing tissue with a high work requirement would be governed by a system in which peroxidase activity predominated over catalase activity and that the reverse would be true for non-growing tissue. Any change from a non-growing to a rapidly growing state would be accompanied by a decrease in catalase activity and an increase in peroxidase activity. Apparently, this hypothesis is at least qualitatively correct for the change accompanying the germination of cottonseed and for similar changes for a number of other seeds (8, 10, 11).

It cannot be presumed that the ratios of catalase to peroxidase activities as determined on extracts and suspensions of seeds and seedlings reflect quantitatively the ratio of the activities of these two enzymes *in vivo*. Nevertheless, it may be assumed that the above-described *in vitro* measurements reflect trends which are occurring in intact seeds.

It is possible that one of the functions of catalase, if not its most important function in biological oxidation, is to regulate, in conjunction with peroxidase, the amount of free energy of oxidation available for biological work. Changes in catalase-peroxidase ratios may be one of the means by which tissues can smoothly adjust to a change in work requirements as they proceed, for example, from an embryonic to an adult stage or from a non-growing to a germinating stage. Catalase and peroxidase are probably only one of a number of sets of enzymes which compete for the same substrate and which may influence the efficiency of utilization of energy in living systems.

Summary

1. It was found in an investigation of the changes in concentration of catalase and peroxidase in germinating cottonseed that the amount of extractable catalase decreased and that of peroxidase increased during germination. In one experiment, the amount of extractable catalase decreased to 18% of its value in resting seed whereas the

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[4] Keilin and Hartree (7) have suggested that one of the biological functions of catalase is in the catalysis of coupled oxidation of alcohols by means of hydrogen peroxide formed in a primary oxidation. The distribution and extent of such coupled oxidations in nature, however, is as yet undetermined.

extractable peroxidase increased 21-fold in the course of a 5 day period of germination.

2. The above-mentioned changes occur in both the radicle and the hypocotyl and cotyledons of the cotton seedling.

3. The decrease in amount of extractable catalase during germination was observed to be much greater than the decrease in total catalase.

4. It is suggested that one of the functions of catalase is to regulate, in conjunction with peroxidase, the amount of free energy of oxidation available for biological work.

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The Catalytic Effect of Metal Ions on Alcoholysis of the Penicillins

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INTRODUCTION

It was found at an early stage of the chemical investigations on the penicillins that their alkali salts, when dissolved in primary alcohols, completely lost their biological activity in the course of a few hours (1).

The first experiments were carried out with a mixture of penicillins containing predominantly Δ^2-pentenylpenicillin, but benzylpenicillin was found to behave in the same way. The loss of biological activity is accompanied by a decrease of optical activity (2), and it was established that the reaction products formed were α-monoesters of β-alkali salts of the penicilloic acids (3). The reaction between alcohols and the penicillins is, therefore, an alcoholysis proceeding according to the scheme:
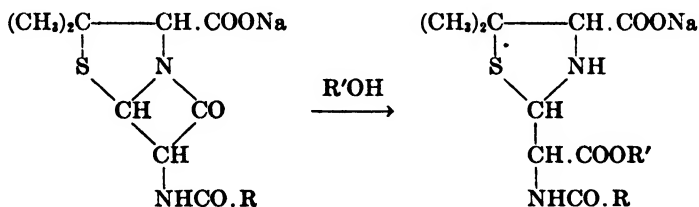
In contradistinction to the alkali salts of Δ^2-pentenyl- and benzylpenicillin, both of which were found to react readily with methanol, it was stated that the sodium salt of p-hydroxybenzylpenicillin possessed a much higher stability in this solvent. It was found to preserve its activity unchanged after standing for as long as 48 hours at room temperature in methanol; under comparable conditions sodium benzylpenicillin had lost ⅔ of its antibacterial power after 18 hours (4,5).

The higher stability of p-hydroxybenzylpenicillin in methanol has always been difficult to understand on the basis of its chemical structure.

It appeared possible to utilize the difference in the stability in methanol of p-hydroxybenzyl- and the other penicillins for the development of a differential determination method for p-hydroxybenzylpenicillin in mixtures. Such a method was needed for the characterization of the penicillins produced by different mold strains. Consequently, when a pure sample of sodium p-hydroxybenzylpenicillin was made available, through the courtesy of Dr. F. Stodola of the Northern Regional Research Laboratory, Peoria, Ill., its stability, alone and in mixtures with sodium benzylpenicillin, was studied under different conditions.

In the course of this work observations were made which did not agree with the statements in the penicillin literature, and these led to a general reinvestigation of the stability of the penicillins in alcohols. The results of this investigation are reported below.

EXPERIMENTAL

Material and Methods

The biological activity of the penicillins was determined by the cylinder-plate test (6). All assays were made in triplicate. Crystalline sodium benzylpenicillin, prepared in this laboratory from a commercial product (activity about 700 units) of the Distillers (Biochemicals) Co., Speke, and crystalline sodium p-hydroxybenzylpenicillin were used throughout the work. The dimercaptopropanol was a pure preparation made by Dr. L. Stocken in the Department of Biochemistry, Oxford.

Comparison of Rates of Inactivation of Sodium Benzyl- and Sodium p-Hydroxybenzylpenicillin in Methanol

The first comparative experiments showed that sodium p-hydroxybenzylpenicillin, far from being more stable than sodium benzylpenicillin, was always more rapidly destroyed in methanol. A representative experiment is shown in Table I. (See also Tables IV and VIII.)

. TABLE I

Inactivation of Sodium Benzyl- and Sodium p-Hydroxybenzylpenicillin in Commercial Methanol

Type of penicillin	Activity in units/ml. after incubation at 37°C. for	
	0 hours	20 hours
Benzyl	500	230
p-Hydroxybenzyl	500	0

Experiments were carried out in which the temperature, the concentration of the reagents, the water content of the methanol, and the samples of the methanol, were varied to ascertain whether the differences between these results and those of the American authors (4,5) were due to any of these factors, but in all cases sodium p-hydroxybenzylpenicillin was inactivated more rapidly than sodium benzylpenicillin.

Catalytic Effect of Metal Ions on the Rate of Inactivation of the Penicillins in Alcohols

In the course of these experiments it was noticed that lower concentrations of the sodium penicillins (5 units/ml.) were inactivated much more rapidly by methanol than were higher concentrations (500 units/ml.) (Table II). In view of the fact that a large

TABLE II

Inactivation of Sodium Benzylpenicillin in Different Concentrations in Methanol

Initial concentration in units/ml.	Activity in units/ml. after incubation at 37°C. for	
	4 hours	20 hours
500	470	230
5	0	

excess of methanol was present in both cases (500 units of sodium benzylpenicillin correspond to about 0.3 mg.) the result was unexpected, and led to the suspicion that impurities in the methanol might be responsible for the concentration effect, and might play an important part in the mechanism of the inactivation process. In accordance with this assumption, it was found that careful distillation of the methanol led to a considerable increase in the life of sodium benzylpenicillin in this solvent (Table III). When the nature of the impurities responsible for the accelera-

TABLE III

Inactivation of Sodium Benzylpenicillin in Stock and Distilled Methanol

Methanol	Activity in units/ml. after incubation at 37°C. for		
	0 hrs.	8 hrs.	24 hrs.
Stock	500	0	
Distilled	500	400	230 or less

tion of the methanol inactivation was considered, water and aldehydes were excluded, and the attention of the authors was turned to the role of metal ions, which had been found to accelerate the inactivation of penicillin salts in aqueous solution (1). It was, in fact, found that additions of small amounts of copper ions (in the form of

copper sulphate) to distilled methanol greatly accelerated the destruction of sodium benzyl- and sodium *p*-hydroxybenzylpenicillin in this solvent (Table IV). Of other metal ions tested zinc and tin (added as zinc sulphate and stannous chloride) were found to be particularly active (Table V). Spectroscopic analysis of samples of commercial methanol (kindly carried out by Dr. G. Mannkopf, Rothamsted Experimental Station, Harpenden) showed that it contained significant amounts of both tin and copper ions. The tin could be eliminated by distillation, but it proved much more difficult to remove the last traces of copper by this method.

TABLE IV

Inactivation of Sodium Benzyl- and Sodium `p-Hydroxybenzylpenicillin in purified Methanol by Added Copper Ions

Type of penicillin	Conc. of Cu in γ/ml.	Activity in units/ml. after incubation at 37°C. for			
		0 hrs.	2 hrs.	4 hrs.	8 hrs.
Benzyl	0	500	410	480	475
	1	470	450	390	0
p-Hydroxybenzyl	0	550	400	0	
	1	535	170	0	

TABLE V

Inactivation of Sodium Benzylpenicillin in Distilled Methanol by Zinc, Tin, and Copper Ions

Metal ions	Concentration of metal in γ/ml.	Activity in units/ml. after incubation at 37°C. for		
		0 hrs.	6 hrs.	24 hrs.
Zn	0.1	540	415	0
	0.5	500	0	0
Sn	0.1	515	300	0
	0.5	490	0	0
Cu	0.1	430	445	345
	0.5	445	390	0

The effect of the metal ions is catalytic in nature. Thus, one atom of zinc is capable of bringing about the inactivation of about 50 molecules of sodium benzylpenicillin in 24 hours. The sodium salts of the penicillins are much more stable in secondary and tertiary alcohols than in primary alcohols. However, inactivation can be brought about by addition of small amounts of metal ions (Table VI).

TABLE VI

Inactivation of Sodium Benzylpenicillin in Secondary and Tertiary Butanol by Added Metal Ions

Alcohol	Metal ion	Concentration of metal in γ/ml.	Activity in units/ml. after incubation at 37°C. for		
			0 hrs.	6 hrs.	24 hrs.
sec.-Butanol	Zn	5	490	275	0
	Sn	5	450	475	350
	Cu	5	515	460	425
tert.-Butanol	Zn	5	555	465	0
	Sn	5	500	475	420
	Cu	5	630	535	460

TABLE VII

Inactivation of Sodium Benzylpenicillin (500 units/ml.) in Purified Methanol by Copper, Zinc, and Tin Ions, and Protection against this Inactivation by Dimercaptopropanol

Metal	Concentration of metal in γ/ml.	Conc. of dimer-captopropanol in mg./ml.	Hours of incubation at 37°C.	Activity in units/ml.
Cu	1	0	8	0
	1	0.1	8	480
	5	0.1	24	420
Zn	1	0	8	0
	10	0.1	8	500
	10	0.1	24	500
Sn	1	0	6	0
	10	0.1	6	510
	10	0.1	24	330

Retarding Effect of Dimercaptopropanol on Metal-Catalyzed Inactivation of Penicillin Salts by Primary Alcohols

Dimercaptopropanol (also known as "British Anti-Lewisite" BAL) (7, 8) combines with mercury, copper, arsenic, zinc, tin, and other metal ions to give stable com-

pounds of the type

$$
\begin{array}{l}
\text{H}_2\text{CS} \\
\quad\quad\searrow \\
\quad\quad\quad\quad\text{M} \quad\quad\quad\quad\text{(M = metal ion)} \\
\text{HCS}\nearrow \\
\quad| \\
\text{H}_2\text{COH}
\end{array}
$$
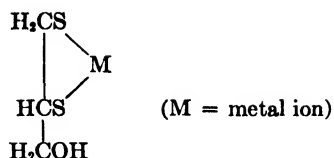
Addition of dimercaptopropanol to methanol resulted in the complete protection of sodium benzylpenicillin on incubation at 37°C. for 24 hours in the presence of relatively high concentrations of metal ions (Table VII). Quite small amounts of dimercaptopropanol are sufficient to stabilize sodium benzylpenicillin, and also the more sensitive p-hydroxybenzylpenicillin, in commercial distilled methanol in the absence of added metal ions (Table VIII).

TABLE VIII

Minimal Concentrations of Dimercaptopropanol Protecting Sodium Benzyl- and Sodium p-Hydroxybenzylpenicillin (500 units/ml.) against Inactivation in Purified Methanol

Type of penicillin	Conc. of dimer-captopropanol in γ/ml.	Activity in units/ml. after incubation at 37°C. for	
		4 hrs.	24 hrs.
Benzyl	0		270
-	1.2		470
p-Hydroxybenzyl	0	0	
	0.6	500	
	10		510

The inactivation of penicillin salts by other primary alcohols can also be prevented completely by addition of small amounts of dimercaptopropanol. Thus, sodium benzylpenicillin did not appreciably lose activity after incubation at 37°C. for 24 hours in n-butanol in the presence of a small amount of dimercaptopropanol (Table IX). Distillation of alcohols over dimercaptopropanol greatly reduces their inactivating action on penicillin salts (Table X).

TABLE IX

Inactivation of Sodium Benzylpenicillin in n-Butanol and Protection by Dimercaptopropanol

Conc. of dimercapto-propanol in mg./ml.	Activity in units/ml. after incubation at 37°C. for		
	0 hrs.	6 hrs.	24 hrs.
0	500	0	
0.1	500	475	460

TABLE X

Inactivation of Sodium Benzylpenicillin (500 units/ml.) in n-Butanol Before Distillation, After Distillation, and After Distillation in the Presence of Dimercaptopropanol

Quality of n-butanol	Activity in units/ml. afer incubation at 37°C. for	
	6 hrs.	24 hrs.
Stock	0	
Distilled	400	0
Distilled over dimercaptopropanol	500	440

Several other metal complex-forming substances were tested for their power to retard the inactivation of penicillin salts by methanol in the presence of metal ions. Thioglycollic acid was much less effective; diethyldithiocarbamate and 8-hydroxyquinoline themselves caused inactivation.

DISCUSSION

The two suggested formulae for the penicillin molecule which received most attention were the "thiazolidine-oxazolone" (I) and the "β-lactam" structure (II). The latter was eventually proved to be correct by X-ray crystallographic measurements (9).
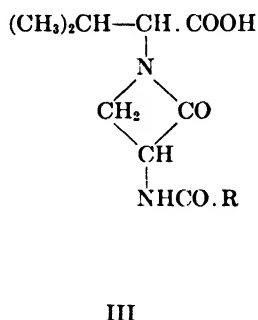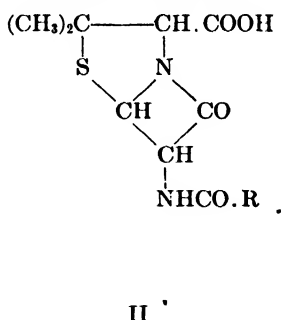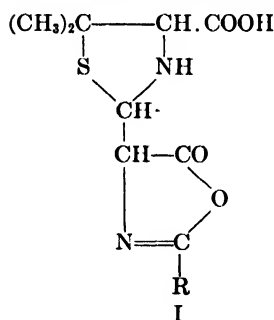
One of the main arguments in favor of structure I was the supposed extreme instability of the penicillin salts in methanol, which was considered to be in accordance with the presence of an oxazolone ring in the penicillin molecule. The relative stability of the penicillin salts in metal-free methanol removes the basis of this argument.

When the mechanism of the inactivation of the penicillin salts by alcohols is considered, it appears unlikely that the alcoholysis occurs through direct fission of the β-lactam ring, as the desthiopenicillins (III) are completely stable in methanol, even after refluxing for pro-

longed periods. As the metal ions which catalyze the alcoholysis of the penicillin salts are known to react with the sulphur atom in thiazolidine rings, it is probable that the alcoholysis is the consequence of their interaction with the sulphur in the penicillin molecule, leading to the opening of the thiazolidine ring. The resulting electronic displacements would tend to break the –CO–N– linkage of the β-lactam ring with the formation of an unstable intermediate oxazolone which would readily undergo alcoholysis to the penicilloate. The strong tendency of the penicillin molecule to rearrange itself into an oxazolone is illustrated by the facile isomerization of methyl benzylpenicillin to methyl benzylpenicillenate under the influence of mercuric chloride. The mechanism of the catalysis of the alcoholysis of penicillin salts by metal ions can be represented by the following scheme:
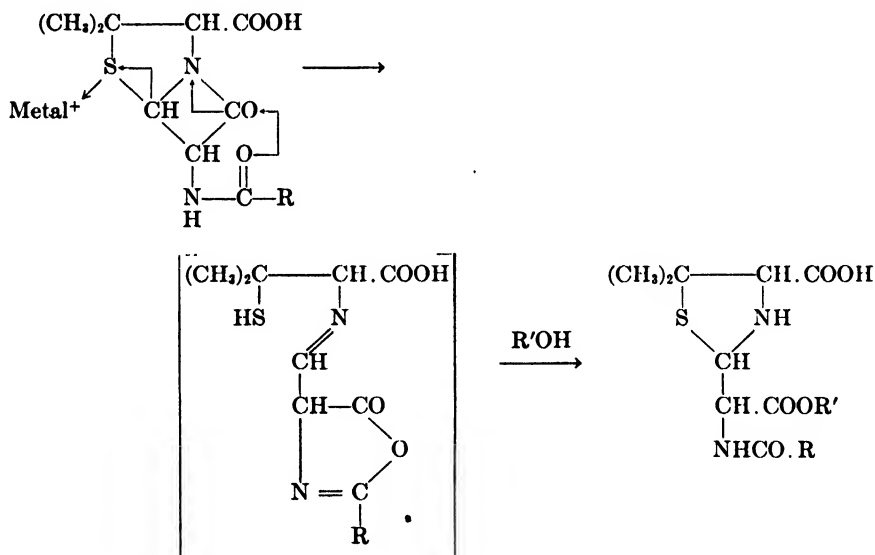
The penicillins can be inactivated by boiling methanol in the presence of dimercaptopropanol. This reaction is probably also initiated by the fission of the thiazolidine, and not the β-lactam ring.

The results recorded above have a practical implication. At present the only practicable way to obtain dry preparations of the alkali salts of the penicillins without loss of activity, is by the process of freeze-drying which involves expensive equipment. The observations recorded above show that it should now be practicable, using ordinary

vacuum distillation methods, to evaporate to dryness solutions of penicillin salts in alcohols which have been freed from metals by suitable treatment.

SUMMARY

1. Sodium *p*-hydroxybenzylpenicillin is less stable in methanol than sodium benzylpenicillin.

2. Metal ions greatly accelerate the alcohol inactivation of penicillin salts. Zinc, tin and copper are particularly active in this respect.

3. The inactivation of the penicillins by alcohols can be greatly retarded by the addition of small amounts of dimercaptopropanol.

4. The stability of the penicillins in alcohols freed from traces of metal ions is further evidence against the presence of an oxazolone ring in the penicillin molecule, and is in agreement with the β-lactam structure.

5. The stability of the penicillins in alcohols freed from traces of metal ions makes it possible to obtain dry preparations of the alkali salts of the penicillins by evaporation to dryness of alcoholic solutions by simple vacuum distillation.

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The Influence of Aeration and Agitation on the Yield, Protein and Vitamin Content of Food Yeasts[1]

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INTRODUCTION

That aeration plays an important role in the manufacture of yeast is a well-established fact. Effective aeration depends on a number of factors, such as amount of air, fineness of air dispersion, rate of agitation, size of the fermenter, and the volume of the medium. The most efficient use of air is obtained when the air particles are small and remain in contact with the liquid for the longest period of time. In practice, aeration is commonly expressed as l. of air/l. of medium passed through the liquid/min. (l./l./m.).

Pavcek *et al.* (1) supplied air at the rate of 1.7 l./l./m. and obtained 10 times higher yields of *Saccharomyces cerevisiae* than in non-aerated medium. Stark *et al.* (2) studied the propagation of *Saccharomyces cerevisiae* in 500 ml. of medium contained in flasks under different methods of air dispersion, *viz.*, open glass tube, Aloxite stone and Berkefeld candle (N). Considering the amount of air used and the density of yeast cells obtained, aeration by Berkefeld candle (N) was the most efficient and that by glass tube the least efficient. Unger *et al.* (3) supplied air at the rate of 1.8 l./l./m. to grow distillers' yeast on a pilot plant scale. Thaysen *et al.* (4) obtained a cell count of 1200×10^6/ml. of *Torulopsis utilis*, which is about 3 times that reported by Unger *et al.* with the same supply of air. More recently Feustel and Humfeld (5) have devised a new laboratory fermenter employing a high speed agitator for air dispersion and obtained a population of approximately 4 billion cells (48 mg. dry yeast)/ml., which is probably the highest so far reported. The yield of yeast, based on sugar supplied, however, was not increased by the high agitation rate. Kurth (6) observed that with *Torula utilis* the fermentation time could be reduced from 72 hrs. to 16 hrs. by providing aeration with fritted glass tubes instead of by shaker. For the large scale production of *T. utilis*, German operators (7) used 20–30 l. of air/g. of yeast

produced in a Vogelbusch type fermenter. The air was supplied from the compressors to a hollow perforated propeller to increase the efficiency of aeration. For the production of *T. utilis* from wood hydrolyzates in a Waldhof type of laboratory fermenter (agitation, 900 r.p.m.) Harris *et al.* (8) used about 240 cu. ft. of air/lb. of yeast (15 l./g.).

Agitation seems to have received much less consideration than aeration. Weleminsky and Butschowitz (9) obtained similar yields in aerated wort and unaerated, but rapidly circulated, wort. De Becze and Liebmann (10) claimed to have obtained 3–4 times larger yields by agitating the mash with CO_2 or N_2 than in absence of agitation. A 3- or 4-fold increase over that obtained in still medium, however, would not be equal to that given by an aerated medium. A typical figure for anaerobic yeast fermentation is about 5% of the sugar fermented. A 3- or 4-fold increase, therefore, is less than half of that usually obtained in an aerated medium.

Pavcek *et al.* (1, 11) reported that yeasts grown under anaerobic conditions had a much higher thiamine content than those grown under aerobic conditions. Lewis *et al.* (12), in confirming the above findings, observed that the thiamine content of *T. utilis* was doubled when the yeast was grown anaerobically. It should be noted, however, that the overall yield of thiamine is less under anaerobic than under aerobic conditions because the production of yeast in the unaerated media is very low. Burkholder (13) reported that the synthesis of riboflavin by *Candida guilliermondia* was doubled by agitation.

It was the purpose of this paper to study the effect of aeration and agitation, not only on yield and protein content, but also on the vitamin composition of the yeasts.

EXPERIMENTAL

Yeast Strains

Four strains of yeasts, namely, *Saccharomyces cerevisiae* No. 53, *Torulopsis utilis* No. 3, *Candida arborea*, and *Oidium lactis* A, were used. The details regarding the origin and use of these cultures are given in Fiat Final Report No. 499 (7) and in our previous paper (14).

Inoculum

The inoculum was grown on the medium recommended by Peterson *et al.* (15), at 30°C. in a shaker. Starting from the stab culture, yeast was successively inoculated into 10 ml., 100 ml. and finally into 10 liters of the medium. In each case 5% of the 18 hrs.-old culture was used.

A 10% inoculum was used for growing the yeast.

Fermentation Medium

Molasses. Two kinds of beet molasses, from Mason City, Iowa, and Ovid, Colorado, and one kind of cane molasses, Hawaiian blackstrap, were used. Beet molasses was used without any treatment, but the Hawaiian cane molasses was clarified by corn steep liquor treatment as previously described (14).

Medium. The fermentation medium contained molasses equivalent to approximately 1% sugar, $(NH_4)_2HPO_4$ equal to 0.05% concentration and corn steep liquor corresponding to 0.33% solids. The pH of the medium was adjusted to 4.6.

Fermentation

The fermentation was carried on in a 25 l. Pyrex glass jar. This was placed in a steel frame and closed with a steel cover fitted air-tight on the jar by means of screws attached to the frame. The cover was equipped with aeration and agitation facilities. Air, sterilized by passage through a cotton filter, was supplied through a sparger at the bottom of the jar in the form of fine bubbles. Just above the sparger ring, a four blade propeller kept the medium well agitated. A baffle plate attached to the air line prevented coning and produced a turbulent motion of the liquid. A detailed description of the construction and operation of the fermenter is given elsewhere (16).

Foaming and Its Control

So much foaming was encountered with *C. arborea* and *O. lactis* that 3% octadecanol in lard oil proved quite incapable of controlling it. "Vegifat-Y" was more effective. Ten ml. of "Vegifat-Y" was sufficient for 3 of the yeasts, but, in case of *C. arborea*, 20–25 ml. was required.

Analytical Methods

The dry weights of yeasts were obtained after centrifugation and drying in an oven at 100°C. Reducing sugar was determined by the micro method of Shaffer and Somogyi (17), and nitrogen by the Kjeldahl method. Thiamine was determined by the thiochrome method of Conner and Straub (18), riboflavin by the microbiological method of Strong and Carpenter (19), niacin by that of Krehl *et al.* (20), and folic acid by that of Roberts and Snell (21).

The vitamins were determined on wet and washed samples immediately after fermentation except those in Table VII where the determinations were made on dried yeast samples.

RESULTS AND DISCUSSION

Data for Yeasts Grown on Mason City Molasses

S. cerevisiae, T. utilis, and *C. arborea* were grown on Mason City beet molasses under varying conditions of aeration and agitation. The effect of these factors on the rate of sugar utilization, yield, protein, and vitamin content is summarized in Fig. 1 and Tables I, II, and III.

Rate of Sugar Utilization

In Fig. 1 the rate of sugar utilization by *T. utilis* under different conditions of aeration and agitation is plotted against time. It is evident from the figure that the sugar utilization was independent of

TABLE I

Effect of Aeration and Agitation on Yield, Protein and Vitamin Content of S. cerevisiae[a]

(Mason City Beet Molasses)

Aeration	Agitation	Yield on S.F.	Protein in D.M.	Vitamins, γ/g. D.M.			
				Thiamine	Riboflavin	Niacin	Folic acid
l./l./m.	*r.p.m.*	*Per cent*	*Per cent*				
2.4	0	31.0	57.3	41.3	66.6	323.2	34.1
3.6	0	36.5	56.2	37.2	62.4	376.8	25.1
0	500	20.8	57.3	52.7	84.1	239.5	23.7
0	750	19.6	55.4	52.8	91.3	209.8	26.4
0.1	250	34.7	56.4	38.6	63.6	346.9	24.8
0.1	450	39.7	54.9	35.9	60.0	407.6	25.6
0.4	240	39.3	56.7	40.9	61.7	407.0	27.1
1.2	350	42.3	54.2	34.7	56.3	439.3	21.7

[a] S.F. denotes sugar fermented; D.M., dry matter; r.p.m., revolutions per minute; l./l./m., liters of air per liter of medium per minute.

TABLE II

Effect of Aeration and Agitation on Yield, Protein and Vitamin Content of T. utilis

(Mason City Beet Molasses)

Aeration	Agitation	Yield on S.F.	Protein in D.M.	Vitamins, γ/g. D.M.			
				Thiamine	Riboflavin	Niacin	Folic acid
l./l./m.	*r.p.m.*	*Per cent*	*Per cent*				
0	0	3.7	52.1	53.0	108.4	212.0	15.7
0.6	0	7.7	52.4	59.5	104.7	230.4	18.7
1.8	0	22.0	52.0	51.2	97.1	288.2	14.1
3.6	0	33.5	49.5	55.5	90.0	296.5	18.1
0	333	13.1	49.3	41.4	100.1	276.6	18.5
0	500	24.2	.54.1	29.6	91.5	290.4	21.3
0	750	62.2	51.3	23.1	66.5	409.9	7.1
0.15	240	40.7	47.8	31.4	95.7	301.6	16.5
0.15	480	59.5	42.0	24.7	64.3	399.0	13.0
0.60	270	60.4	38.8	23.7	64.2	394.2	10.1
0.60	345	63.4	49.8	30.8	64.0	399.8	8.2
0.60	480	57.9	39.4	21.2	67.5	321.1	10.7
0.60	800	64.0	47.3	20.3	63.4	410.4	9.4
1.80	375	58.1	49.6	21.9	65.1	361.7	6.8
1.80	750	66.2	48.4	17.4	62.5	475.0	7.8

TABLE III

*Effect of Aeration and Agitation on Yield, Protein and Vitamin
Content of C. arborea*

(Mason City Beet Molasses)

Aeration	Agitation	Yield on S.F.	Protein in D.M.	Vitamins, γ/g. D.M.			
				Thiamine	Riboflavin	Niacin	Folic acid
l./l./m.	*r.p.m.*	*Per cent*	*Per cent*				
0	0	7.6	42.4	42.9	95.2	316.2	40.6
1 0	0	19.6	42.7	38.6	81.6	316.6	24.3
2.4	0	37.8	40.6	29.2	70.0	327.1	30.6
3.6	0	50.0	40.2	20.2	60.6	350.7	26.4
0	500	19.8	43.1	29.7	82.5	316.6	21.9
0	750	20.0	40.7	31.0	81.1	317.5	21.4
0.15	250	32.0	40.9	21.4	70.0	325.4	16.7
0.15	500	69.2	41.3	14.4	58.4	372.9	16.2
0.60	250	74.9	40.4	13.2	55.8	375.9	16.7
0.60	500	71.9	39.5	12.8	61.6	369.9	18.3
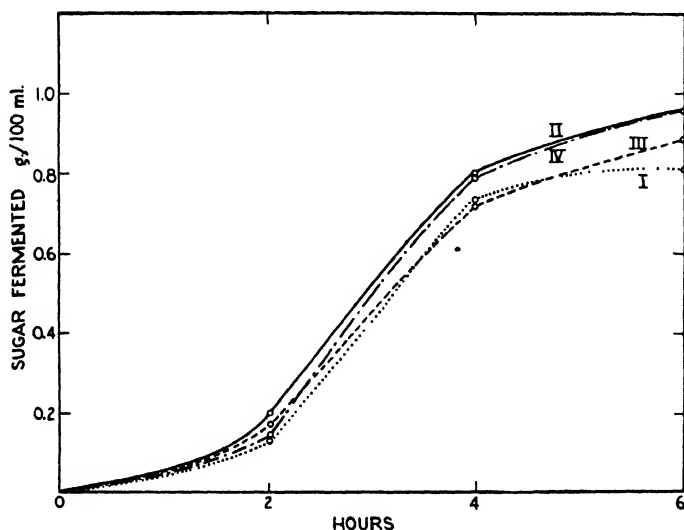
FIG. 1. The rate of sugar utilization by *T. utilis* grown on Mason City beet molasses. Aeration, l./l./m.; agitation r.p.m.; yield as percent of sugar fermented in order: I. 0,0, 3.7; II. 3.6, 0, 33.5; III. 0, 700, 62.2; IV. 0.6, 480, 57.6.

aeration and agitation. Similar results were obtained with *S. cerevisiae* and *C. arborea*. In all 3 yeasts during the first 2 hours, the sugar utilization was slow, perhaps because the organisms required some time to adapt themselves to the new environment. Within 4 hours nearly 90% of the reducing sugar present was consumed. The rate of sugar utilization after 4 hours was very slow which may be due to the presence of unfermentable or slowly fermentable residual sugar in molasses.

Yield of Yeasts

From Table I it is evident that the maximum yield of *S. cerevisiae* could not be obtained with agitation alone. Aeration alone, or agitation and aeration together, gave much higher yields. As against this, agitation alone proved quite effective in giving maximum yield of *T. utilis*, while aeration failed to do so (Table II). This may be due to an increased supply of oxygen to the cells, or to the removal of the byproducts from the surface. Still another explanation may be that agitation breaks off the budding cells sooner and affords them greater surface. Approximately maximum yields (60%) of *T. utilis* were obtained with agitation at a rate of 270 r.p.m. and aeration rate of 0.6 l./l./m. Reducing the air supply to one-fourth and approximately doubling the agitation rate gave an equivalent yield. *C. arborea* differed from *S. cerevisiae* and *T. utilis* in that its maximum yield could not be obtained with aeration or agitation alone (Table III). Low aeration (0.15 l./l./m.) required a high agitation (500 r.p.m.) to give approximately maximum yields, *i.e.*, about 70%. When the agitation was cut in half (250 r.p.m.) and the aeration increased 4-fold (0.6 l./l./m.) a slightly higher yield (75%) was obtained. The same inverse relationship between aeration and agitation was observed with *S. cerevisiae* and *T. utilis*.

Protein Content

With all the yeasts there was no appreciable effect of aeration and agitation on the protein content of the yeasts. As rich sources of proteins, the 3 yeasts fall in the following descending order: *S. cerevisiae*, *T. utilis*, and *C. arborea*.

Vitamin Content

From Tables I, II, and III it is evident that thiamine and riboflavin decreased with an increase in yield, while niacin increased with an in-

crease in yield. Thus, there seems to be an indirect relationship between vitamin content and various conditions of aeration and agitation. Changes in folic acid content were irregular. As a source of vitamins, *S. cerevisiae* was richest in thiamine and folic acid. *C. arborea* was poor in thiamine and niacin but rich in folic acid, while *T. utilis* was poor in folic acid. Riboflavin values were nearly the same in all 3 yeasts.

It should be pointed out that the total yield of vitamins/g. of sugar fermented increased markedly with an increase in the yield of the cells. For instance, with *T. utilis* (Table II), when the yield was 3.7%, the thiamine, riboflavin, niacin, and folic acid produced were, respectively, 1.96, 4.0, 7.8, and 0.58 γ/g. of sugar metabolized, but corresponding values increased to 11.5, 41.5, 314 and 5.2 γ when the yield rose to a maximum of 66.2%. These changes serve to point out the difference in yeast metabolism under anaerobic and aerobic conditions, and perhaps reflect the vitamin requirements of the cells in these two types of metabolism.

The figures also indicate the notable capacity of these yeasts for synthesizing vitamins. The synthesis was particularly marked with respect to niacin; 185–314 γ of the vitamin were apparently synthesized /g. of sugar fermented. The vitamin content of the molasses and corn steep liquor samples was very low as compared to the amounts of vitamins present in the yeast cells raised on them. Rogers and Mickelson (22) have also recently shown that steffenized molasses contains only negligible amounts of these vitamins. Of course, more rigorous and conclusive evidence of synthesis can only be obtained by using a glucose-inorganic salts medium. Such a medium would exclude the possibility of degradation products functioning as precursors of vitamins.

FEEDING EXPERIMENTS

The effect of successive additions of molasses during germentation (so called feeding) on the rate of sugar utilization, yield of yeasts and the conversion efficiency of yeasts was then investigated. After 8 hours of fermentation, pH rose to 6–7, which was adjusted to 4.5–5, and then feeding was started. The feed comprised a molasses solution containing 5–10% reducing sugar, calculated as glucose, together with appropriate quantities of nutrients according to the original fermentation medium. At intervals, the sugar present in the fermentation medium, volume of yeast, and the rate of sugar utilization were determined. During the feeding period, pH was controlled between 4.5–5 and sugar concentration was kept between 0.2 and 0.5%. The temperature was held at 30°C. during the entire fermentation. The production of
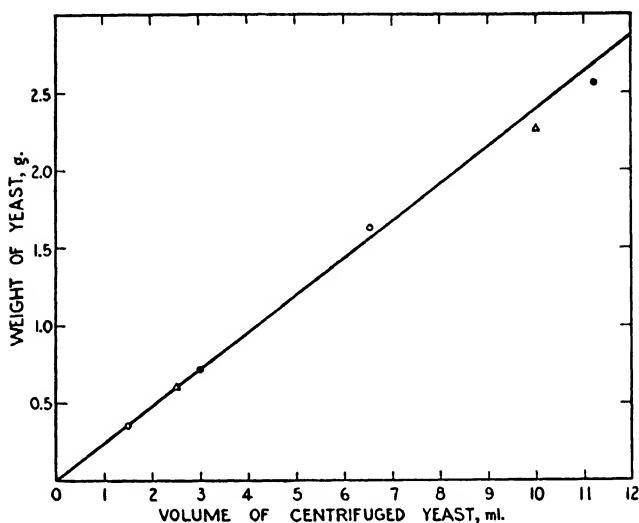
FIG. 2. Relationship between volume of yeast and the dry weight
(*S. cerevisiae* ○—○, *T. utilis* △—△ and *C. arborea* ●—●).

TABLE IV

*Effect of Successive Additions of Molasses during Fermentation[a] on
Yield of S. cerevisiae*
(Mason City Beet Molasses)

Time	Sugar present	Volume of medium	Sugar, as glucose, in added molasses	Sugar consumed, glucose	Weight of dry yeast	Efficiency [b]
hrs.	*Per cent*	*ml.*	*g.*	*g.*	*g.*	
0	0.76	5,000	38		3.88	
8	0.05	5,500	25	35.5	17.31	37.6
9		6,000	25		25.3	
10.5		7,000	50		34.5	
11.5	0.53	7,500	50	100.9	48.3	44
13		8,000	50		60.5	
14	0.31	8,000		208.2	74.2	33.8
15.5		8,500	50			
17		9,000	50			
18	0.29	9,000		306.9	114.0	35.8
19.5		9,500	50			
20.5	0.21	9,500		363.0	134.1	35.7

[a] Aeration 5 l./m., agitation 400 r.p.m.
[b] Weight of dry yeast/100 g. of sugar fermented.

yeast was followed by the periodic determination of volume of yeast, by centrifuging the fermented mash in calibrated centrifuge tubes. It is quicker to obtain volume than dry weights, hence volume of yeast was determined after almost every hour of feeding, while dry weights were determined only after 8 and 20.5 hrs. of fermentation. Volume of the yeast was plotted against dry weight (Fig. 2), which indicated a linear relationship between volume and weight. The dry weight of yeasts/ml. of yeast equals 0.24 g. Approximately a similar relationship between weight and volume oj yeast has been reported by Feustel and Humfeld (5). From the curve, the weights of yeasts were extrapolated. The data are presented in Tables IV, V, and VI. The sugar was most efficiently converted to yeast cells by *C. arborea*, though utilization of sugar was most rapid with *T. utilis*. Yields based on sugar fermented were practically the same for these two organisms during 8 hours of fermentation, but after 12.5 hours of feeding, the percentage yield of *T. utilis* dropped more rapidly than that of *C. arborea*.

The decrease in efficiency in the later period of fermentation has also been reported by Sperber (23) and Feustel and Humfeld (5). Sperber studied the conversion of glucose to *Torulopsis* yeast during different periods of growth with addition of glucose at 2–3 hr. intervals. His average efficiency during 0–9 hrs. was 54.8%. Feustel and Humfeld found that the average efficiency of *T. utilis* during 0–8 hrs. was 44%. There is much irregularity in both cases if different periods of growth are considered individually. None of these workers studied the efficiency after 9–10 hrs. of fermentation. The decrease in efficiency may be explained on the assumption that during the

TABLE V

Effect of Successive Additions of Molasses during Fermentation[a] on Yield of T. utilis

(Mason City Beet Molasses)

Time	Sugar present	Volume of medium	Sugar, as glucose, in added molasses	Sugar consumed, glucose	Weight of dry yeast	Efficiency
hrs.	*Per cent*	*ml.*	*g.*	*g.*	*g.*	
0	0.76	5,000	38	•	5.80	
8	0.05	5,500	25	35.5	28.5	64.1
9		6,000	25		51.0	
10.5		7,000	50		62.0	
11.5	0.13	7,500	50	128.9	88.5	64.0
13		8,000	50		113.0	
14	0.24	8,250	25	213.8	129.0	57.8
15.5		8,750	50			
17		9,250	50			
17.5		9,750	50			
18	0.29	9,750		379.8	201.4	51.3
19.5		10,250	50			
20		10,750	50			
20.5	0.24	10,750		482.2	235.0	47.5

[a] Aeration 5 l./m., agitation 400 r.p.m.

TABLE VI

Effect of Successive Additions of Molasses during Fermentation[a] on Yield of C. arborea

(Mason City Beet Molasses)

Time	Sugar present	Volume of medium	Sugar, as glucose, in added molasses	Sugar consumed, glucose	Weight of dry yeast	Efficiency
hrs.	*Per cent*	*ml.*	*g.*	*g.*	*g.*	
0	0.9	5,000	45		4.04	
8	0.06	5,500	25	42	34.5	72.5
9		6,000	25			
10.5		7,000	50			
11.5	0.52	7,500	50	108.6	80.0	60.9
13		8,000	50			
14	0.34	8,000		217.8	138.0	61.5
15.5		8,500	50			
17		9,000	50			
18	0.34	9,000		314.4	186.5	58.2
19.5		9,500	50			
20.5	0.24	9,500		372.2	240.0	63.3

[a] Aeration 5 l./m., agitation 400 r.p.m.

later period of fermentation, a large quantity of sugar goes to maintain non-proliferating cells and not to make new cells. Hence the greatest overall efficiency should be obtained in a short period of fermentation.

The rate of sugar utilization was slower and conversion efficiency was lower with *S. cerevisiae* than with the other two yeasts, but there was no appreciable change in efficiency of conversion during the feeding period.

O. lactis proved to be the most inefficient in the utilization of sugar. This might be due to its thick mycelial growth which prevented uniform distribution of air. After 15.5 hrs. fermentation, the whole mass became so thick that it was necessary to dilute the medium. The cells could not be properly centrifuged and no direct relationship between the volume of the yeast on centrifugation and its dry weight was found to hold.

Similar feeding experiments using these four yeasts were conducted with Ovid beet and Hawaiian cane molasses. Space does not permit a detailed discussion of the data, but it will probably suffice to say that in general, the results were similar to those obtained with Mason City molasses.

Effect of Kind of Molasses

To see whether the kind of molasses has any influence on the rate of sugar utilization, yields, protein and vitamin contents of yeasts, 3 kinds of molasses, two beet and

one cane were used. The above-mentioned 4 organisms were grown under the optimum conditions of aeration and agitation. The results are presented in Table VII. Approximately 90% of the sugar in the molasses was fermented in all cases. As expected with an aeration rate of 1 l./l./m. and an agitation rate of 400 r.p.m., maximum yields of the 4 yeasts were obtained with the 3 kinds of molasses.

The protein content of the yeasts grown on different kinds of molasses was the same and was in the same order as discussed previously (Tables I and III).

Comparing the yields of dry yeasts and their protein content on the basis of the weight of sugar in molasses, there seems to be no marked difference in the 3 kinds of molasses used. However, if comparisons are based on the weight of molasses, Ovid beet molasses is distinctly poor, because of its rather low reducing sugar content (14).

The effect of the kind of molasses on the vitamin content of the yeast was irregular. In some instances the beet molasses seemed to be superior to cane (e.g., thiamine in S. cerevisiae) but in others, the reverse appeared to hold (e.g., niacin in S. cerevisiae and T. utilis). In several examples, the vitamin data of the yeasts raised on the two lots of beet molasses differed markedly. Hence, any generalization regard-

TABLE VII

Yields, Protein and Vitamin Content of Yeasts Grown under Optimum Conditions of Aeration and Agitation[a]

Yeasts	Yield on S.F.	Protein in D.M.	Vitamins, γ/g. D.M.			
			Thiamine	Ribo-flavin	Niacin	Folic acid
	Per cent	*Per cent*				
Mason City beet molasses						
S. cerevisiae	41.0	45.5	37.1	43.7	296.0	33.6
T. utilis	72.0	49.3	26.3	52.5	213.0	9.6
C. arborea	74.0	41.3	22.2	57.5	301.0	11.5
O. lactis	74.1	39.3	14.4	40.0	195.6	12.3
Ovid Colorado beet molasses						
S. cerevisiae	44.2	42.5	31.4	39.1	276.9	35.6
T. utilis	76.0	47.5	22.1	46.2	221.6	10.1
C. arborea	70.9	39.3	16.4	46.2	157.1	18.5
O. lactis	72.0	38.5	12.2	40.2	185.6	12.0
Hawaiian cane molasses						
S. cerevisiae	38.0	50.6	27.7	45.0	402.0	34.0
T. utilis	74.2	43.7	20.7	53.7	282.4	14.6
C. arborea	71.1	41.8	16.1	60.0	312.5	19.6
O. lactis	69.2	46.2	12.0	41.2	194.6	14.8

[a] Aeration 1 l./l./m., agitation 400 r.p.m.

ing the effect of molasses on vitamin content is not warranted by the present data.

SUMMARY

The effect of aeration, agitation, or combinations of the two, and the kind of molasses used as substrate, on the rate of sugar utilization, yields, protein and vitamin content of *Saccharomyces cerevisiae*, *Torulopsis utilis*, *Candida arborea*, and *Oidium lactis* was investigated.

With the first 3 yeasts, sugar utilization was similar and independent of aeration, agitation or the kind of molasses. Within 4 hours nearly 90% of the reducing sugar of molasses was fermented. *Oidium lactis* was a slow fermenter of sugar, perhaps because its thick mycelial growth prevented good aeration of the medium.

The yield of these yeasts varied from 4 to 75% based, on sugar fermented. Nearly maximum yield of *S. cerevisiae* was obtained with aeration alone, of *T. utilis* with agitation alone, while both aeration and agitation were required for maximum yield of *C. arborea*.

The protein content of the yeasts remained almost unchanged under all conditions.

The figures for vitamins varied markedly with variation in yields. Thiamine and riboflavin decreased, while niacin increased with increase in yield. Folic acid figures varied irregularly with yield. The yeasts showed a marked capacity of synthesizing vitamins, which was particularly outstanding in the case of niacin.

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Amino Acids in Lupine and Soybean Seeds and Sprouts [1]

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INTRODUCTION

Earlier studies[3] have emphasized the distribution in plants of aspartic acid, glutamic acid and the mono amides of these amino acids. Following the isolation of asparagine in asparagus shoots by Vauquelin and Robiquet (4) in 1806, glutamic acid in wheat gliadin by Ritthausne (5) in 1866, and glutamine in beetroot by Schulze and Bosshard (6) in 1883, the dicarboxylic amino acids in the seeds, sprouts, leaves and proteins of many types of plants have been determined by isolation and chemical procedures. Other amino acids have not been investigated extensively because convenient methods for their determination have not been generally available.

Since the newly developed microbiological procedures are well suited to the determination of amino acids in plant materials, it seemed worth while to undertake investigations on the amino acid composition of plant seeds and of sprouts grown under different conditions. The lupine and the soy bean were selected for the initial experiments because the lupine was studied extensively by early workers, particularly Ritthausen and Schulze, and both the lupine and the soy bean are important foods.

EXPERIMENTAL

Seeds of *Lupinus angustifolius*[4] and of soy bean (Illini M13)[5] were soaked overnight in distilled water and planted in moist sand in a light-tight cabinet and in moist garden soil out of doors. Sand, rather than water, culture was employed since it was found in preliminary experiments that excess moisture led to rapid growth of molds· and early decomposition of the sprouts. The sand was washed thoroughly with distilled water before planting the seeds and it was kept moist with distilled water during the growth of the sprouts. Plant nutrients other than water were not added although it seems unlikely that all traces of minerals and other nutrients were removed from the sand by the described treatment.

The sand planted with lupine seeds was marked in 7 equal areas and, at two-day intervals, the 50 largest and sturdiest sprouts were harvested from each of the areas in turn.[6] This procedure was employed to insure the selection of uniformly developed sprouts at each growth period. The entire plants were taken except for small amounts of root fibers which adhered to the sand and the seed coats which dropped from the cotyledons of sprouts older than 3 days. The soy bean sprouts were harvested in a similar manner but only after a 12-day growth period. Representative whole plants of lupine and soy bean grown out of doors in the light were taken after 48 days. Whole lupine (90 days) and whole soy bean plants (136 days) were also harvested after flowers and fruit had formed. In all cases, the plants grown both in the light and dark appeared to be free from mold.

Immediately after harvesting, the plants were freed from extraneous material, weighed, and immersed in 7 N hydrochloric acid. The suspension was refluxed for 44 hours on an oil bath, and excess acid was removed by distillation *in vacuo*. A refluxing time longer than that commonly employed for the hydrolysis of purified proteins was used because of the relatively slow digestion of the plant material. These hydrolysis conditions were considered to be satisfactory since it had been shown previously (11) that there was no significant destruction of any of the 12 amino acids determined in the present experiments when they were refluxed for 24 hours with 9 N hydrochloric acid in the presence of excess carbohydrate and fat. The residual material was extracted thoroughly with boiling distilled water and the extracts were filtered, cooled and diluted to a known volume. Aliquots of this solution were taken for the determination of arginine (12), aspartic acid (13), glutamic acid (14), glycine (15), histidine (16), isoleucine (17), leucine (17), lysine (18), methionine (19), phenylalanine (20), threonine (21), and valine (17) by the indicated microbiological methods.

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[4] Purchased from C. A. Simpson, Monticello, Florida. The growth of the lupine, a seed-producing winter legume, has been described by Warner (7).

[5] Obtained through the courtesy of Dr. W. C. Rose, U. of Illinois.

[6] The plants were allowed to grow only for 15 days since at that time the stalks began to droop and soften and the roots began to darken and fall off, probably because of exhaustion of the nutrients furnished by the seeds. Vickery and Pucher (8) reported that intense decomposition of asparagine invariably began after 12 days culture of *Lupinus angustifolius* seeds in the dark, but Merlis (9) and Lugg and Weller (10) found that asparagine continued to increase up to 18 days growth of the etiolated seedlings.

Discussion

The primary purpose of the present experiments was to investigate the changes in total (free and combined) amino acids occurring during the growth of the lupine and the soy bean in moist sand in the dark and in garden soil under natural conditions. Consideration is to be given in later studies to the distribution of amino acids in the free and combined states as well as to the influence of nutrients on the amino acids in growing plants. Although aspartic acid is present in sprouts largely as asparagine, the latter was hydrolyzed to aspartic acid and determined in this form in the present work. No microbiological procedure is available for the differentiation of aspartic acid and asparagine.

As shown in Table I, the mg. of nitrogen and the mg. of total amino acids determined per seed and per sprout were essentially constant

TABLE I
Amino Acids in Seeds and Etiolated Sprouts of Lupinus Angustifolius[b]

Amino acid		Mg. per seed or sprout						
	Seed	Sprouts, age in days						
		3	5	7	9	11	13	15
Arginine	4.7	4.5	3.2	2.8	2.6	2.2	2.1	1.9
Aspartic acid	5.2	8.4	14.7	18.0	22.0	24.0	24.8	25.5
Glutamic acid	11.8	10.1	6.4	4.3	3.4	2.4	1.5	1.5
Glycine	2.0	2.1	1.6	1.6	1.5	1.3	1.1	1.0
Histidine	1.4	1.8	1.4	1.4	1.5	1.4	1.5	1.4
Isoleucine	2.8	2.4	2.1	1.8	1.7	1.3	1.1	0.89
Leucine	3.4	3.7	3.0	2.4	2.4	1.8	1.7	1.3
Lysine	2.5	2.5	2.1	2.0	2.0	1.9	1.7	1.6
Methionine	0.20	0.24	0.22	0.20	0.21	0.18	0.16	0.15
Phenylalanine	2.2	1.9	1.8	1.7	1.8	1.6	1.5	1.4
Threonine	1.9	1.3	1.3	1.3	1.3	1.2	1.1	1.0
Valine	2.3	2.2	2.0	2.0	2.1	1.7	1.7	1.4
Total amino acids	40.6	41.1	40.0	39.6	42.4	40.9	40.0[a]	39.0
Total weight (mg.) per seed or sprout	173	536	1048	1416	1608	1556	1668	1620

[a] Value assumed since it could not be calculated owing to loss of some hydrolyzate.

[b] The average total nitrogen per seed was 8.94 mg. and per sprout was 8.36 mg. The seeds contained 2.62% ash, 9.38% moisture and 5.17% nitrogen.

during the 15-day growth of the lupine in the dark. During this period, the aspartic acid increased from about 5 mg. to 25 mg. which was equivalent to an increase from about 13 to 65% of the total amino acids determined. There occurred, simultaneously, a decrease in glutamic acid from about 12 mg. to 1.5 mg. and decreases in the other amino acids ranging from about 80 to 40% of the quantities present in the seeds. It may be significant that the seeds and the etiolated sprouts had about the same histidine content. Similar results were obtained with soy bean (Table II) although the total amount of the 12 amino acids per seed was about 30% greater than that per sprout.

TABLE II

Amino Acids in Seeds and Etiolated Sprouts of Soy Bean (Illini M13) [a]

Amino acid	Seed mg.	Sprout, 12 day mg.
Arginine	3.1	1.7
Aspartic acid	6.1	14.2
Glutamic acid	8.7	2.1
Glycine	1.9	1.0
Histidine	1.2	1.0
Isoleucine	. 3.2	1.7
Leucine	3.7	2.0
Lysine	3.5	1.6
Methionine	0.6	0.34
Phenylalanine	2.4	1.4
Threonine	2.5	1.2
Valine	2.6	1.7
Total amino acids	39.4	29.9
Total weight (mg.) per seed or sprout	135	950

[a] L. B. Rockland collaborated in these experiments. The average total nitrogen per seed was 7.66 mg. and per 12-day sprout was 7.48 mg. The seeds contained 4.52% moisture, 4.81% ash, and 5.51% nitrogen.

There was marked increase in the absolute and relative amounts of most of the amino acids determined in lupine sprouts grown in the light under natural conditions although the increase for aspartic acid was not greater than that for other amino acids (Table III). The proportion of histidine in the 48-day sprouts was somewhat less, and in the 90-day sprouts was somewhat greater, than that in the seeds. It is noteworthy that the lupine seeds and the 48-day sprouts contained nearly the same absolute amounts of arginine and glutamic acid. The distribution of

amino acids in soy bean and soy bean sprouts was analogous to that in the lupine.

It may be concluded from these results that aspartic acid (asparagine) is synthesized during the growth of the lupine and the soy bean from the other amino acids, especially glutamic acid, in these beans.

TABLE III

Amino Acids in Plants of Lupinus Angustifolius and Soy Bean (Illini M13) Grown in Light

Amino acid	Lupinus angustifolius				Soy bean			
	Mg. per sprout		Per cent of total amino acids determined		Mg. per sprout		Per cent of total amino acids determined	
	48 days	90 days	48 days	90 days	48 days	90 days	48 days	136 days
Arginine	5.2	18.5	6.5	6.0	7.2	44	6.9	6.0
Aspartic acid	12.2	51.7	15	17	22	174	20	24
Glutamic acid	11.3	45.1	14	15	15	106	14	15
Glycine	5.2	23.6	6.5	7.6	5.8	44	5.5	6.1
Histidine	2.0	11.5	2.6	3.7	2.9	22	2.8	3.0
Isoleucine	7.2	23.7	9.0	7.6	8.4	45	8.0	6.3
Leucine	9.2	34.4	12	11	12	72	11	9.9
Lysine	7.6	29.7	9.5	9.6	8.4	65	7.9	9.0
Methionine	1.5	5.8	1.9	1.9	2.3	13	2.2	1.9
Phenylalanine	5.9	20.8	7.5	6.7	7.4	44	7.0	6.1
Threonine	5.2	20.4	6.6	6.6	5.9	41	5.6	5.7
Valine	7.0	25.8	8.7	8.3	8.6	54	8.1	7.4
Total amino acids	79.5	311			106	723		
Total weight (mg.) per sprout	3.92	20.8			3.31	21.9		

It has been shown previously, by Schoenheimer *et al.* (22), that disproportionately large amounts of the isotopic nitrogen of an amino acid fed to a rat was regenerated as aspartic and glutamic acids in the body protein. Results "closely analogous to previous observations on the metabolism of proteins in animals" were obtained by Vickery *et al.* (23) in their studies on the tobacco plant.

SUMMARY

Twelve amino acids have been determined by microbiological methods in seeds, etiolated seedlings, and plants of *Lupinus angustifolius* and soy bean (Illini M13). The most marked changes which occurred in etiolated seedlings grown in moist sand in the dark were increase of aspartic acid (asparagine) and decrease of all other amino acids except histidine. Total amino acids of plants grown in garden soil under natural conditions increased although the individual amino acid changes relative to the other amino acids were less than those which occurred in the dark.

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A Coenzyme for Phosphoglucomutase

A study of the enzymatic transformation of galactose-1-phosphate revealed that extracts of *Saccharomyces fragilis* will transform this substance into a reducing ester only in the presence of a thermostable factor. The same factor has been found to be necessary for the conversion of glucose-1-phosphate into glucose-6-phosphate with extracts of *S. fragilis* or *S. cerevisiae*.

The action of this coenzyme can be revealed in crude maceration extracts, but these retain a considerable activity in the absence of added coenzyme. Results recorded in Fig. 1 were obtained with a
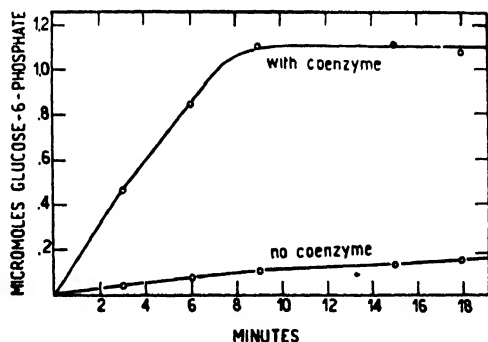
FIG. 1. Activation of phosphoglucomutase with the coenzyme. Reducing power measured with a copper reagent and a glucose-6-phosphate standard. Incubation at 30°C. of: partially purified enzyme, 0.005 μM Mg^{++}, synthetic glucose-1-phosphate 1.5 μM, purified coenzyme containing 0.1 μM total phosphate. Total volume 0.2 ml.

brewers' yeast enzyme partially purified by ammonium sulphate fractionation and dialysis. The coenzyme preparation was obtained from brewers' yeast, the ratio: activity/extinction at 260 mμ was 150 times higher than in the extract obtained by heating the yeast in one volume of water and filtering. The ratio activity/total phosphate was 20 times higher.

In Table I are recorded the changes in the phosphate fractions and in reducing power. These correspond to those known to be brought about by phosphoglucomutase.

We have been unable to identify this factor with any of the known coenzymes. It can be precipitated from crude solutions with lead, mercury, silver, and barium salts. In general, it follows inorganic phosphate during fractionations with these reagents. Inorganic phosphate can be removed from it as magnesium ammonium or as uranyl salts, when the coenzyme will no longer precipitate with mercury salts.

TABLE I

Changes in Phosphate Fractions

Results in micromoles. Conditions similar to those in Fig. 1. Half the amount of coenzyme.

Coenzyme	Time	P Inorg.	P Labile[a]	P Stable[b]	Reduction[c]
	min.				
With	0	0.04	0.89	0.07	0.00
With	15	0.08	0.09	0.83	0.86
Without	0	0.05	0.89	0.01	0.00
Without	15	0.08	0.85	0.07	0.01

[a] Phosphate liberated in 10 min. at 100°C. in 1 N acid minus inorganic.

[b] Total phosphate minus labile and inorganic.

[c] Reducing power in terms of a glucose-6-phosphate standard.

Purified preparations are colorless and show ultraviolet absorption at 260 mμ, but purification will have to be carried on further to find out whether this absorption is due to the active compound.

Treatment with $N/2$ acid at 100°C. will destroy the activity in 15 min. It is more resistant to treatment with alkali under the same conditions.

Kendal and Stickland (1) obtained an activation of phosphoglucomutase by adding hexose diphosphate, but Cori *et al.* (2) were unable to obtain any effect. The hexosediphosphate preparation of Kendal and Stickland may have been contaminated with the new coenzyme reported in this paper.

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Purification and Crystallization of Human Pancreatic Amylase

In the course of our work on amylolytic enzymes, the purification and crystallization of α-amylase of pig pancreas (1–4), of human saliva (5), and of *Bacillus subtilis* (6) have been described.

Although having the same enzymatic action, these enzymes are not identical chemical substances, for their solubility in water and their affinity for the substrate are different.

A comparison of the two animal amylases with that of human pancreas seemed to be of interest, especially in answering the main question presented: Does the amylase of human pancreas resemble an enzyme from another gland of the same species (human saliva), or is it more closely related to the enzyme from the same gland of another species (pig pancreas)? To answer this question crystalline human pancreatic amylase has been prepared.

The starting material was obtained from the Pathological Institute[1] of this University. After dissection, the glands are immediately put into acetone at $+2°C.$, then stocked at $-20°C.$ All subsequent operations are carried out between $0°$ and $2°C.$

Preparation of the Dry Powder. The finely minced glands are defatted by successive extraction with acetone, then with ether, and finally dried *in vacuo.* The powder thus obtained is stable.

Extraction. The powder is extracted with 15 times its weight of 0.5 M sodium acetate for 48 hours.

Purification. The purification is effected in 7 steps: two fractional precipitations with acetone, retaining the precipitate between 46 and 64% acetone (I) and between 46 and 69% acetone (II). Then two precipitations with ammonium sulfate solution brought to pH 8.0 by the addition of ammonia and saturated at 0°C. At the first

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[1] We are much obliged to Prof. E. Rutishauser for the facilities afforded.

precipitation the salt solution is added up to 0.425% saturation (III), the next time to 0.250% saturation (IV). In the dissolved product the sulfate ion is then exchanged for acetate ion by treating 3 times with Amberlite IR-4B previously charged with an excess of sodium acetate and washed (V). Subsequently the solution is shaken twice at pH 7 with a mixture of chloroform and amyl alcohol according to Sevag (VI), and finally precipitated with acetone up to 70% (VII).

Crystallization and Recrystallization. The precipitate is dissolved in the least possible volume of 0.01 N NH$_4$OH and the solution brought to pH 6.4–6.6 by addition of N acetic acid. After standing for a week, 75% of the enzyme is deposited in a crystalline form. The crystals are washed with water, suspended in 0.1 N NH$_4$OH, and gently shaken for 24 hours (final pH 11). After spinning down undissolved crystals, the solution is brought to pH 6.4–6.6 by means of N acetic acid.   The enzyme crystallizes either in fine needles or in elongated plates (Fig. 1).
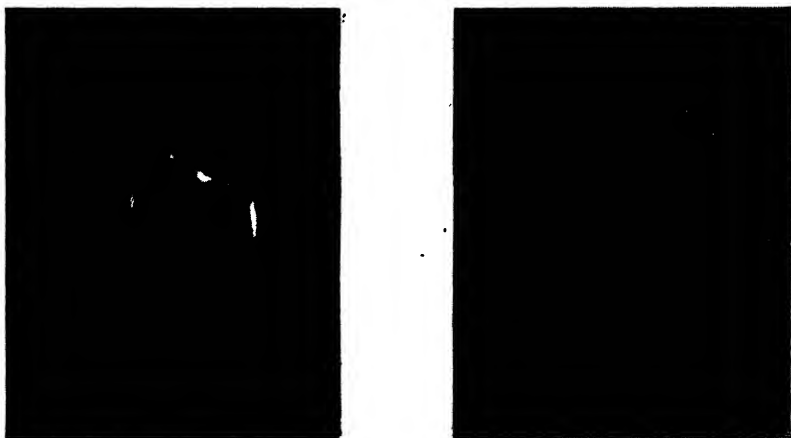
FIG. 1. Crystalline human pancreatic amylase.

Properties. The enzyme gives the typical protein reactions. Its activity is 4.3×10^3 mg. of maltose/mg. nitrogen (2). The enrichment is 25-fold with respect to the crude extract and the yield is 25%. The solubility in water is extremely low at pH 7 and 8.5; it dissolves readily in water at pH 11. In this medium the enzyme is very stable.

Comparison with Other Animal Amylases. It gives the same kind of crystals and has, under the conditions mentioned above, the same solubility as human salivary amylase, whereas pig pancreatic amylase is about 100 times more soluble at pH 8.5. The quotient enzymatic activity/weight of pure enzyme is the same for both human amylases; it is considerably higher than the quotient for pig pancreatic amylase.

We therefore conclude that both human amylases are identical chemical substances.

Our findings are thus analogous to Bonnichsen's (7) results which indicate the identity of horse liver and horse blood catalase and a difference between horse blood and human blood catalase.

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Book Reviews

Experimental Embryology. M. W. WOERDEMAN AND CHR. P. RAVEN. Monographs on the Progress of Research in Holland. pp. 132; 40 figs. Elsevier Publishing Co., Inc. New York and Amsterdam, 1946. Price $2.50.

This monograph reviews the work done in experimental embryology in Holland during the war, and more specifically in the anatomical-embryological laboratory in the University of Amsterdam and in the laboratory of general biology of the State University of Utrecht.

From the former laboratory comes a series of experimental studies on amphibian eggs; these include the determination of the lens-primordium in frog larvae, the determination of the polarity of the ectoderm, studies of the development of teeth in Triton and Amblystoma, of the development of the pro- and meso-nephrons in Axolotl embryos, of limb induction and of the effect of X-rays on the blastulae of Axolotl, as well as biochemical investigations on amphibian eggs (measurements of respiration and studies of the effects of trivalent arsenic, of carcinogens, and of decapsulation). To these are added the results of experiments on avian embryos, particularly in connection with the development of the gonads.

From the University of Utrecht come reports on various phases of the development of the pond snail, *Limnea stagnalis L.* The mechanism of oviposition, the egg-laying stimulus, oogenesis, cytoplasmic processes in the uncleaved egg, as well as physical changes in the egg, centrifugation experiments, and the action of various ions, are considered in short sections each a few pages long. A concluding section describes experiments on amphibian eggs (determination of the neural crest, development of the pineal organ, determination of the lateral plate and genital ridges in urodeles, *etc.*)

The material of the monograph is presented very clearly and is excellently illustrated. The conditions under which this work was accomplished are described in a preface which should be read by all who believe that scientific discovery depends materially on the success of financial drives and the conveniences which no doubt result therefrom. "On account of the electric current being cut off in the autumn of 1944 the electrically heated operating room and incubators for the work on birds' eggs could not be used any more after that date. . . . Also part of Trampusch's work could not be continued, as the X-ray apparatus stopped working owing to lack of current. . . . With the means at our disposal we meantime succeeded in fitting up a simple laboratory provided with what is absolutely necessary for research work on metabolism. In this department Ten Cate started work with great diligence, but the unfavorable times have hindered him very much in his work. All kinds of apparatus, simple glassware, chemicals were soon no more available, and finally work became quite impossible when the electric current and gas supply failed. . . . From the above mentioned it should be evident with what difficulties work was attended during the years of war. Nevertheless we are glad that we have been able to remain active for a long time."

ERIC PONDER, Mineola, Long Island, N. Y.

Biochimie et Médecine. By L. MASSART, Professor in the Faculty of Medicine of the University of Ghent. Editions "Erasme" S. A., Brussels, 1947. Paper-bound. 71 pp. Price 200 francs.

This short monograph, in the series of "Actualités Médicales," is designed to present some essential problems of biochemistry and their recent developments in relation to medicine, and to indicate present-day trends in medical biochemistry. Since its function is not that of a text, no extensive literature citations are given.

Its scope may be indicated best by a listing of the chief chapter headings: the use of isotopes, enzymes, vitamins, vitagens (the term suggested by Rosenberg) and hormones, intermediary metabolism, nutrition, and frontiers in biochemistry. The subjects discussed are well chosen and the presentations are up-to-date. Some topics, chosen more or less at random, will indicate this, brief discussions of hyaluronidase, hypertensinase, the chemical origin of the thyroid hormone and action of thiouracil, the citric acid cycle, insulin and hexokinase, enrichment of cereal products, the recommended dietary allowances of the Food and Nutrition Board of the National Research Council. Among the frontiers, borderline fields, histochemistry, chemotherapy, antibiotics and competitive inhibitors, and transmethylation are discussed.

The presentation, although of necessity brief, includes most of the newer developments of biochemistry and should be of particular value to physicians of those parts of Europe in which the opportunities to be informed of newer trends in biochemistry have been limited, or lacking entirely, in the last few years.

HOWARD B. LEWIS, Ann Arbor, Mich.

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Annual Review of Microbiology, Vol. I. Edited by C. E. CLIFTON, S. RAFFEL and H. A. BARKER. Annual Reviews, Inc., Stanford, California, 1947. vi + 404 pp. Price $6.00.

This volume represents the newest addition to the established and valuable Annual Review family. The launching of this new branch of the family comes appropriately when there is taking place a rapid development of the constellation of disciplines now subsumed by the name microbiology. It is particularly valuable that an Annual Review should appear in this field where so many, and often previously separated, sciences now are fusing.

The preface to the volume states as objectives of the Review to give—

"annual reviews of the more active fields of research, leaving more quiescent areas to be summarized as developments demand. . . . It is intended that the reviews will be critical appraisals rather than mere compilations of the literature. This implies that an individual reviewer may neglect some worthwhile contributions as not pertinent to his development of a topic. By soliciting the services of different reviewers for the same subject in succeeding years and with consequent variations in approach, the undesirable aspects of such treatment will be largely offset."

This editorial outlook augurs well for the future.

The present volume contains 17 articles: Morphology and cytology of protozoa (D. H. Wenrich, pp. 1–10); Antigenic variation in protozoa and bacteria (J. A. Harrison, pp. 19–42); Life cycle of malaria parasites (C. G. Huff, pp. 43–60); Variation in phytopathogenic fungi (C. M. Christensen, E. C. Stakman and J. J. Christensen, pp. 61–84); Variation in phytophathogenic viruses (L. O. Kunkel, pp. 85–100); Some

aspects of the problem of growth factors for protozoa (A. Lwoff, pp. 101–114); Bacterial metabolism (D. D. Woods, pp. 115–140); Nitrogen metabolism (E. F. Gale, pp. 141–158); Industrial fermentations (M. J. Johnson, pp. 159–172); Quaternary ammonium compounds (O. Rahn and W. P. van Eseltine, pp. 173–192); Antibiotics (R. G. Benedict and A. F. Langlykke, pp. 193–286); Chemotherapeutic agents (E. M. Lourie, pp. 237–262); Immunochemistry (H. P. Treffers, pp. 263–290); Some aspects of active immunization (J. Freund, pp. 291–308); Medical and epidemiological aspects of enteric infection (A. J. Weil, pp. 309–332); The Rickettsiae (N. H. Topping and C. C. Shephard, pp. 333–350); Respiratory viruses (T. Francis, Jr., pp. 351–384). There is an author index (pp. 385–398) and a subject index (pp. 399–404).

It would not be true to say that all the above articles achieve the objectives set forth in the editorial preface. The writing of critical reviews is a very time-consuming task and all those who undertake it are entitled to the sincere gratitude of their fellow workers. At the same time, each reviewer must realize that the task is onerous and be prepared to do the hard work that the responsibility entails. The task is severe in microbiology but is the more worth doing well since this is a field where the critical review has a particularly valuable and positive role to play. Most of the articles in this volume maintain a good standard; all are packed with information, although this is better digested and synthesized in some than in others.

In implementing the objectives of the Review as quoted above the Editors and Editorial Committee have a cardinal role to play in choosing topics to be reviewed and appropriate reviewers. It is to be hoped that one of their main aims will be to help weld the different disciplines into a coherent body of knowledge. In this first volume of the new series the Editors and Editorial Committee have made a good beginning in what should prove to be one of the most useful members of the Annual Reviews group.

B. C. J. G. Knight, Kent, England

Introduction to Carbohydrate Biochemistry, 2nd ed. By D. J. Bell, B.Sc., M.A., Ph.D., F.R.I.C., University Lecturer in Biochemistry, Cambridge University. Tutorial Press, Ltd., Clifton House, Euston Road, London, N.W. 1, England. viii + 108 pp. Price, 6s.

The period which has passed since the first edition of this book has shown great advancement in our knowledge of the utilization and transformation of carbohydrates in biological systems. It is gratifying that the author chose to completely rewrite and expand the chapters in the earlier work relating to the field in which he has made notable contributions. He has succeeded in presenting the subjects of phosphorolysis, fermentation and glycolysis so concisely and clearly that students of biochemistry can grasp the fundamentals of this phase of carbohydrate chemistry with a minimum amount of work. His treatment of energy-rich phosphate bonds and collateral subject matter is particularly noteworthy.

To make room for the new presentations, the chapters on photosynthesis and bacterial metabolism of the first edition were omitted. The chapters on the sugars, polysaccharides, oxygen glycosides, and uronic acids have undergone little change, except for the addition of discussions of recent work on starch and glycogen. A concise treatment of the nitrogen glycosides and nucleotides provides an excellent background for the chapters dealing with the synthesis and breakdown of carbohy-

drates in biological systems. The topics are introduced in an elementary manner and are developed skilfully. Brevity, while desirable for the student in need of a rapid review, sometimes leads to over-simplification. Thus, on p. 9 the statement appears that glycosides "are all alkali-stable . . . unless the substituting radical belongs to certain classes of phenols." Actually, alkali-sensitive glycosides which do not contain phenolic groups are known. On p. 11 is the statement: "One of two conditions must be satisfied in order that a stable furanose derivative can exist. (A) The active H of the reducing group must be substituted by glucoside formation. (B) The hydroxyl, engagement of the adjacent O-ring with which would give a pyranose, must be substituted, e.g., fructofuranose derivatives:." These conditions will not account for the furanose structures of glucurone, mannurone, and of crystalline lactulose. On p. 19, the statement that pectin is "Built from D-galacturonic radicals" leaves the student ignorant as to the presence of the acetyl, methyl, galactose, and arabinose groups present in this substance. Also, on p. 20 no mention is made of periodate oxidation in the list of methods for investigating polysaccharide structure. Necessarily, many phases of carbohydrate chemistry are not covered, but, all in all, much useful knowledge is included in a small space. There are no literature references in the text and the general reading references at the end of the chapters are largely confined to review articles and textbooks. The work is well illustrated with exceptionally clear structural formulas.

This book is recommended for all persons who desire a concise, orderly summary of present knowledge concerning the synthesis and breakdown of carbohydrates in biological systems.

HORACE S. ISBELL, Washington, D. C.

The Pathology of Nutritional Disease. By R. H. FOLLIS. Charles C. Thomas, Springfield, Illinois, 1948. vii + 291 pp. Price $6.75.

This concise summary with 791 references affords an excellent review of a limited area of nutrition research during the past 30 years. The discussion is devoted largely to the nutrition of the rat, with limited consideration of a few other species such as man, the dog, and common farm animals. Little attention is devoted to the foreign literature, except for some discussion of English and Australian research. Thus, the life-long work of Agduhr, the Swedish histologist, is not even mentioned in the sections concerned with vitamin E and muscle degeneration. The extensive literature concerning chickens, turkeys, fish, and other vertebrates is excluded. Likewise, little attention has been given to the numerous publications from experiment stations. The field of gerontology is never mentioned. The author has confined his discussions largely to that which appeared in the older biochemical journals and the Journal of Nutrition. Hence, it is familiar to well-trained graduate students in nutrition. This text, however, should prove useful to young medical graduates or specialists in sciences, other than nutrition, who wish to familiarize themselves with one phase of current American research. The work is very readable. The illustrations are excellent.

CLIVE M. McCAY, Ithaca, New York

Detoxication Mechanisms. By R. TECWYN WILLIAMS, Senior Lecturer in Biochemistry, University of Liverpool. John Wiley and Sons, Inc., New York, N. Y., 1947. viii + 288 pp. Price $5.50.

The difficult task of bringing together a bewildering mass of data on the fate of organic compounds in the animal body has been admirably accomplished by Dr. Williams. The main purpose of this systematic survey of the data was to see if any definite rules governing the transformations of organic compounds *in vivo* could be worked out on the basis of the facts already known, and, if so, could one predict the path of an organic compound in the animal organism, as well as its effect on the organism in the course of such a transformation. According to the author such predictions "must rest upon analogies from experience gained by studying closely related compounds and knowledge concerning the degree of specificity of known (and unknown) enzyme systems of the body. Such enzyme systems may vary from species to species both qualitatively and quantitatively, and this variation may contain the key to an explanation of why a compound follows different metabolic routes in different species of animals."

The author makes no claim to finality of judgement on any phase of metabolic transformations whenever the available data do not permit final evaluation. Still, suggestions and hypotheses are freely made which make the book stimulating reading. Problems which await solution are plainly outlined. Inconsistencies and contradictions are pointed out along, with well digested references to the bibliography. The impartial treatment of the entire subject will be of interest and of equal appeal and value to biochemists, pharmacologists, enzymologists, and all those who are interested in what happens to an organic compound in the animal organism and what happens to the animal in the course of metabolism of the compound.

The book is divided into 14 chapters, including an introduction and a chapter covering theoretical considerations and conclusions, a bibliography, and an index.

The text is relatively free from misprints and serious inaccuracies. Only a few could be mentioned. On p. 61, Dr. Williams reviews the species of animals which were shown to be capable of synthesizing mercapturic acids, and specifically excludes the pig as the species which cannot carry out this synthesis. The pig can safely be returned to the species of mercapturic acid synthesizers, as it has been demonstrated that this animal can synthesize the corresponding mercapturic acids from bromobenzene and naphthalene (*J. Biol. Chem.* **113**, 675 (1936)). On p. 84, a statement is made that dibenzyl thioether gave rise to hippuric acid in the rat. Instead of dibenzyl thioether the author undoubtedly meant dibenzyl disulfide, as the original publication claimed. On p. 106 Dr. Williams mentions Muenzen, Cerecedo and Sherwin (*J. Biol. Chem.* **67**, 469 (1926)), among others, who were unable to detect any acetyl derivative of *p*-aminobenzoic acid in the urine of dogs and man after the administration of *p*-aminobenzoic acid to these species. On the contrary, these authors were the first to demonstrate the acetylation of *p*-aminobenzoic acid in man.

Dr. Williams stresses the importance of the quantitative aspect of the metabolic fate of an organic compound. No doubt, quantitative information would shed considerable light on the relative importance of metabolic routes which an organic compound follows in the animal body. Unless all of the administered compound is accounted for, no complete picture of its fate is possible. Routes of administration, dietary conditions, dosage—all these factors play a considerable role in determining the quantitative, and even the qualitative, aspect of the fate of an organic compound *in vivo*. In this connection it should be mentioned that Dr. Williams would have done considerable service had he evaluated critically the relative merits of the methods employed in such

quantitative measurements, inasmuch as a large amount of earlier data regarding metabolic paths of organic compounds was secured by circumstantial evidence based on dubious methods. As a result, in many instances, failure to isolate an excretory product from the urine was interpreted to mean that the animal was unable to synthesize the product. In some instances, a rise in the excretion of inorganic sulfate in the urine following the administration of a sulfur-containing compound was claimed to mean its oxidation. And, conversely, a rise in the excretion of the so-called neutral or unoxidized sulfur in the urine after feeding a compound containing sulfur was interpreted to mean, without further experimental proof, that the administered substance was excreted unchanged. In some instances, the administered compound simply "disappeared" since nothing could be traced in the excreta. Such careless interpretation of experimental techniques and unwarranted use of dubious terms in describing the results only obscured the significance of a large body of valuable data.

The book is a welcome addition to a list of good reference texts in the field. In the opinion of the reviewer, the treatise, in the course of time, will undergo progressive expansion with attendant additions, revisions, and labor pains as newer data will become available. It can be only hoped that Dr. Williams will complete the task before him so as to enable the research worker to predict, if only by analogy, the fate of organic compounds in the animal body. Dr. Williams is to be congratulated on a difficult job well done.

<div align="right">J. A. STEKOL, Philadelphia, Pa.</div>

Cold Spring Harbor Symposia on Quantitative Biology. Vol. XII. Nucleic Acids and Nucleoproteins. Edited by M. DEMEREC. The Biological Laboratory, Cold Spring Harbor, L. I., New York, 1947. xii + 279 pp. Price $7.00.

The selection of nucleic acids and nucleoproteins as the topic of the twelfth Cold Spring Harbor Symposium bears witness to the importance of this field. Since a similar symposium had been arranged only a year earlier in Cambridge, some duplication of subjects and speakers was unavoidable. Very few American scientists were able to attend the Cambridge meeting, however, so that a repetition at a place more accessible to the investigators of this continent seems fully justified. The present volume comprises 25 papers, most of them devoted to biological aspects, with only a few stressing biochemical problems. There is one excellent and concise paper, by J. M. Gulland, dealing with the structure of nucleic acids.

Most of the papers are valuable supplements to the articles of the Cambridge symposium, and the scarcity of review papers prevailing during the last decade has now been offset in a very satisfactory way. The majority of the articles are written by investigators who have made outstanding contributions to the field, and few of the papers deserve criticism. One might note that here and there the length of a paper is inversely proportional to the significance of the data reported. Moreover, in two papers the authors literally repeat for several pages their recent publications in scientific journals.

A nice feature of this volume is the inclusion of the discussion of the papers by participants in the symposium. Interesting new viewpoints and interpretations are brought out by this in several instances. Sometimes criticism occurs to the reader perusing the papers, and it is a pleasant surprise then to find this voiced by a com-

mentator in the appendix to the article. Only a few participants abuse the discussions to point out their own important research.

Notwithstanding a few weaknesses this volume seems to be one of the best Cold Spring Harbor reports. A wealth of information is provided, and no investigator concerned with nucleoproteins will be able to dispense with this book.

Mention should be made of the dedication of this volume to the memory of John Masson Gulland who died shortly after the symposium in a tragic accident. His contributions to the chemistry of nucleic acids have made him the leading authority of this specialty during the last decade.

F. SCHLENK, Ames, Iowa.

An Introduction to Industrial Mycology. Reprint of 3rd Edit. By GEORGE SMITH, foreword by HAROLD RAISTRICK. Edward Arnold & Co., Ltd., London. xiv + 271 pp.; 143 figs. 1947.

The author, as a member of a great English research laboratory, has been in charge of a large collection of fungi, including very many of the species and strains important in industry and medicine. He recognizes at the outset that biochemical investigations involving mold activity have been, in the past, dominated by chemists whose training often failed to include even a trace of microbiology. He tacitly admits that industrial leaders who insist upon employing thoroughly competent chemists frequently take up biochemical work involving molds*. Instead of employing men as competently trained in microbiology as the chemists whom they employ, they expect to assign minor chemists as ignorant of biology as themselves to handle the molds, yeasts, or bacteria needed. This book attempts to train such assistants into some sort of ability to handle fungi, instead of frankly advising employers to hire mycologists comparable in basic training to the best chemists in the organization. To such workers the technical phraseolgy based upon the enormous size of the task appears in the author's phrase—*irrational.*

The author himself went through some such experience, but fails to tell us that he has really become a competent mycologist and is able to sling as many long words of Latin and Greek origin as any of us. This book then gives us what he thinks the non-biological beginner must know if he must work in such a laboratory.

The designation of this book as "Third Edition, Second printing" shows that considerable numbers of workers feel the need of help such as is promised here. The reviewer has checked this printing page by page and figure by figure against the first and second editions. It is essentially the same book, with a few changes in illustrations, and expansions in citations of new applications of molds to industry to account for recent progress.

The introduction covers general information about fungi and the definitions of many of the terms to be used later. Most of it is sound. On p. 2, he regards fungi as "more related to animals" than to plants because they require oxygen for metabolism and give off carbon dioxide. Too bad that he never read up on plant physiology. A brief group key to some of the larger groups of fungi is given. These groups are then taken up in a series of chapters: *Zygomycetes, Ascomycetes,* Yeasts, *etc., Fungi Imperfecti, Hyphomycetes, Aspergillaceae, Penicillium, etc.*

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* Note here, the English spell it mould.

Then there are a series of chapters concerning the work to be done: laboratory equipment and technique, physiology of mold fungi, maintenance of a culture collection, control of mold growth, industrial uses of fungi. These are, on the whole, well written. Industrial uses of fungi are listed but practically no discussion of equipment or operation is offered. Only the possibilities are mentioned as a challenge to the worker.

The book is illustrated with 142 photomicrographs (half tones) and one copy of a drawing from Mangin. The author prefers photomicrographs to line drawings for beginners. The photomicrographs are good as far as they go. Having made many of them and seen thousands more, the reviewer doubts whether the beginner will detect diagnostic values between Figs. 93, 94, 96, 102, 103, 104, 105, 106, and 107, or Figs. 82, 85 or 95, if he has actual cultures of the species before him. In other words, in matters of the detail essential to identification many photomicrographs tell exactly nothing. The reviewer can not imagine a value for Fig. 60, 59, or 70 as definite help, from 39, 40, 46, or 92, or specific difference between 50 and 52, or 83 and 84 if you have had the cultures for comparison. He can not find any excuse for labeling any figure *Aspergillus glaucus*. Fig. 109 does not by any stretch of imagination represent typical *Gliocladium roseum*. Nevertheless, a majority of the figures present accessory information of some value. Supplemented by a reasonable number of diagrammatic drawings, they would be much more useful.

Summed up, as an accessory book for reading by an untrained assistant working under supervision in a technical laboratory, it will help him to understand somewhat of the problems of working with molds. If it drives him to an attack upon the real literature of mycology, it has served well. It can hardly be considered a guide to the conduct of investigations or be used to identify any particular mold. It will, however, open the literature of mycology to some who do not have access to the great manuals which must be consulted by those who undertake serious studies.

CHARLES THOM, Long Island, N. Y.

Elsevier's Encyclopaedia of Organic Chemistry. Vol. 13. Tricyclic Compounds. Edited by E. JOSEPHY and F. RADT. Elsevier Publishing Company, Inc., New York and Amsterdam, 1946. 6½ × 10, xxiv + 1265 pp. Cloth. Subscription Price, $78.00; Serial Price $91.00; Single Volume Price, $104.00.

The second volume of Elsevier's Encyclopaedia of Organic Chemistry (Vol. XIII) has now appeared, shortly after Vol. XIV. Vol. XIV, the first of the new work, dealt with tetra- and higher cyclic compounds and was highly praised by such experts in the fields encompassed as Professors Fieser in Cambridge, Cook in Glasgow, and Ruzicka in Zürich. The review of Vol. XIV which recently appeared in this Journal included a discussion of the scope and general system of classification of the new Encyclopaedia. These aspects, therefore, will not be touched upon again. Vol. XIII contains the tricyclic systems, including compounds with carbon bridges and spiro compounds. The important ring systems of fluorene, anthracene and phenanthrene fall within the scope of this volume. It also includes some natural products from the terpene series, and tricyclic compounds of incompletely known structure.

It seems obvious to the reviewer that any discussion of the new book must be carried on in the light of the other existing compendium of this type, Beilstein's Handbuch.

If the Beilstein had never existed, the Encyclopaedia would have been accepted by every organic chemist with great enthusiasm. If there should be some reluctance on the part of some organic chemists to use the Encyclopaedia, this reluctance can only be explained in terms of long usage of Beilstein and thorough acquaintance with it. The fact that the Beilstein has thus far been the only book of its kind cannot mean that it must necessarily be the best or the only one possible, notwithstanding, of course, its undeniable merits. A book of this kind should particularly fulfill three requirements: it should be complete, it should be as up-to-date as possible, and any desired information should be readily found without ambiguity in the method of arrangement. There can be little doubt that the Beilstein is complete for an indicated period of time, and thus fulfills the first requirement. The editors and founders of the Encyclopaedia, Dr. Radt and Dr. Josephy—Miss Josephy was later killed by the Nazis—were instrumental in the compilation of Beilstein. Their close acquaintance with Beilstein and great experience in chemical documentation should serve as a guarantee that the Encyclopaedia will be as complete, as far as available references are concerned, as is Beilstein. The reviewer has checked a few compounds chosen at random and has convinced himself of the above. Only one exception was observed in the limited random sampling. Reference for the absorption spectra of some dihydroxyanthracenes (Moir, 1927) appears in Beilstein, Zweites Ergänzungswerk, but not in the Encyclopaedia. The reason for this omission is not clear as reference to the same paper is made under dihydroxyanthraquinones, whose absorption spectra were also determined by Moir. The Encyclopaedia also appears to omit certain patent references which are contained in Beilstein, and for which there must be a reason.

The second point—up-to-dateness—is the weakest point of Beilstein. It always moved very slowly, and the bulk of Beilstein today only covers the literature up to 1920. Furthermore, although efforts are being made to continue Beilstein, there is no certainty under the present conditions when the next volume will appear. In the Encyclopaedia the literature will be considered until 4 years prior to publication of each volume, which appears to be the main advantage of this work. The delay with the present volume has been due to the war and the invasion of Holland. Supplements are scheduled to appear 10 years after the publication of each volume.

As to the third point, the Beilstein system is highly scientific. There is no ambiguity in the Beilstein system of classification, and there is only one place for each compound. The Encyclopaedia is based on a less rigid system and seems more convenient to handle, although a thorough comparison of the two systems of classification will only be possible after other volumes of the Encyclopaedia have appeared. Since the basis of arrangement in the Encyclopaedia is the structural skeleton, rather than functional groups, as much information pertaining to one ring system is grouped together as is feasible. Thus different functional derivatives of, for instance, fluorene, are condensed in one volume in the Encyclopaedia, whereas they are spread over many volumes in Beilstein. Furthermore, each volume contains a complete formula index (unlike Beilstein) and a subject index, so that it should not take very long to find a given compound. In Vol. XIII there are 182 pages of index, or well over 10% of the total number of pages. The more important systems, such as anthracene, phenanthrene, etc., are furthermore preceded by tables of contents referring to different functional derivatives.

The reference system seems particularly attractive. In Beilstein each reference appears right with the entry, which may be more convenient. In the Encyclopaedia the references are lumped together at the end of small sections and are arranged chronologically according to years. In ·this way the user immediately obtains a historical survey for each subject. Beilstein does not list the years of the references, except for a special *Zeittafel*. Another attractive feature is the detailed survey which precedes the more important systems. These surveys include the occurrence in nature and in technical products, such as coal tar, and the formation from compounds containing fewer or more carbon atoms of the particular system. The same information is available in Beilstein, but not in such an obviously convenient form.

In a circular letter, accompanying the reviewer's copy the editors also point out that the Encyclopaedia lists the actual data for magnetic susceptibilities, absorption spectra, rotatory dispersion and axis ratios, where Beilstein only lists the references. The Encyclopaedia finally contains many more formulae and reaction schemes than does Beilstein. The printing and general appearance of the book are excellent.

A review is not complete if it is only full of praise. The documentation of the electronic structure of anthracene is rather sketchy and important references have been omitted. The electronic structure and resonance energies of phenanthrene are not mentioned at all. And finally, no mention is made of 9-anthroylacrylic acid, which was synthesized in 1923.

One cannot belittle Beilstein; it has been of invaluable service to organic chemistry, will continue to be so, and is remarkably free from errors. On the other hand, there is no reason why it should not be possible to compose an opus which is at least as good as Beilstein, and in some respects perhaps even superior to the latter. The Encyclopaedia promises to be such a work, and it will find its place next to Beilstein and the other great compendia of chemistry. At the present time both are incomplete. The volumes of each which have been published will supplement each other admirably.

ERNST BERLINER, Bryn Mawr, Pa.

Gramicidin Derivatives. II. Toxicity; Effect of Proteins on Hemolytic and Bacteriostatic Activity; Antibacterial Effect *in vivo*

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INTRODUCTION

A previous report from this laboratory (1) described the preparation, and the hemolytic and bacteriostatic properties of a number of gramicidin derivatives. The new compounds had been obtained by the treatment of gramicidin with a variety of reagents under mild conditions. At isobacteriostatic concentrations, determined *in vitro*, they were found to be considerably less hemolytic than the starting material. These gramicidin derivatives were further investigated to decide whether they could be used systemically, in contrast to the highly toxic parent compound.

EXPERIMENTAL

A. Toxicity of Gramicidin and Gramicidin Derivatives

Toxicity was determined by the intravenous injection of propylene glycol solutions of gramicidin and its derivatives into adult white mice. The volume of solvent injected was kept between 1 and 2 ml./kg. body weight, after it was found that 3 ml./kg. produced no symptoms, 5 ml./kg. led to trembling, followed by death in 20% of the animals, and 7 ml./kg. was regularly lethal (10 out of 10 animals died).

In white rats, Seidenfeld and Hanzlik (2) observed no mortality after the intravenous administration of up to 8 ml. propylene glycol/kg. Between 20 and 30% mortality resulted from 9–12 ml./kg., 50% mortality with about 15–16 ml./kg., and 19 ml./kg. were lethal to all animals used. Our results indicate that mice are about 2–3 times as sensitive to propylene glycol as rats, and about as sensitive as rabbits, which have been studied by Seidenfeld and Hanzlik (2).

Special precautions had to be taken to make certain that the small volumes injected left the tail veins of the mice and entered the general circulation. Propylene glycol is quite viscous and usually blocked blood flow at the site of injection unless

the vessels were dilated by previous immersion of the mouse tail for 2–3 min. in a water bath at about 40–42°C. If propylene glycol remained localized at the site of injection, comparatively large quantities of gramicidin could be injected without producing death. Failure of propylene glycol to leave the tail vein led to necrosis of the tails, which became evident several days after the injection. The high value for the LD_{50} of gramicidin on intravenous administration to mice, reported by Anderson *et al.* (3) as 1.5 mg./kg., might be the result of local arrest of most of the injected propylene glycol solution. With the precautions mentioned above, the LD_{50} for gramicidin was found to be 0.03 mg./kg. or 1/50 of the amount recorded in the literature. In most instances, lethal doses produced convulsions immediately after termination of the injection and led to death within 1–2 min.

Experimental data are listed in Table I. As shown in Table II, all gramicidin derivatives were found to be considerably less toxic than the starting material.

TABLE I

Toxicity of Gramicidin for White Mice on Intravenous Administration

Gramicidin injected	Number of animals killed	Number of animals injected	Mortality
mg./kg.			*Per cent*
0.10–0.15	8	8	100
0.05–0.07	8	12	67
0.04	9	12	75
0.03	4	12	33
0.022–0.025	2	8	25

TABLE II

Toxicity of Gramicidin and Gramicidin Derivatives

Substance	Obtained by treatment of gramicidin with	Nitrogen	LD_{50} for mice mg./kg., intravenous
		Per cent	
Gramicidin	——	13.8	0.03
Methylol gramicidin	CH_2O and NaOH	12.5	0.36
R-38	NaOH	13.3	8.5
R-41	NaOH	13.6	4.2
R-42	NaOH	13.4	4.3
R-46	NH_2OH and NaOH	13.9	6.2
R-51	NH_2OH and NaOH	13.6	5.5
R-52	NH_2OH and NaOH	13.9	3.5
R-47	HCl and CH_3COOH	12.1	30
R-48	Iodine	11.1	2.5

B. Effect of Protein on the Hemolytic Activity of Gramicidin and Gramicidin Derivatives

In the hemolytic tests reported previously (1), human red cells which had been washed twice with isotonic NaCl solution were used. Gramicidin showed considerably less hemolytic activity when whole blood was used as test material. The removal of a protective component in plasma by successive washing is illustrated in Fig. 1. For a better
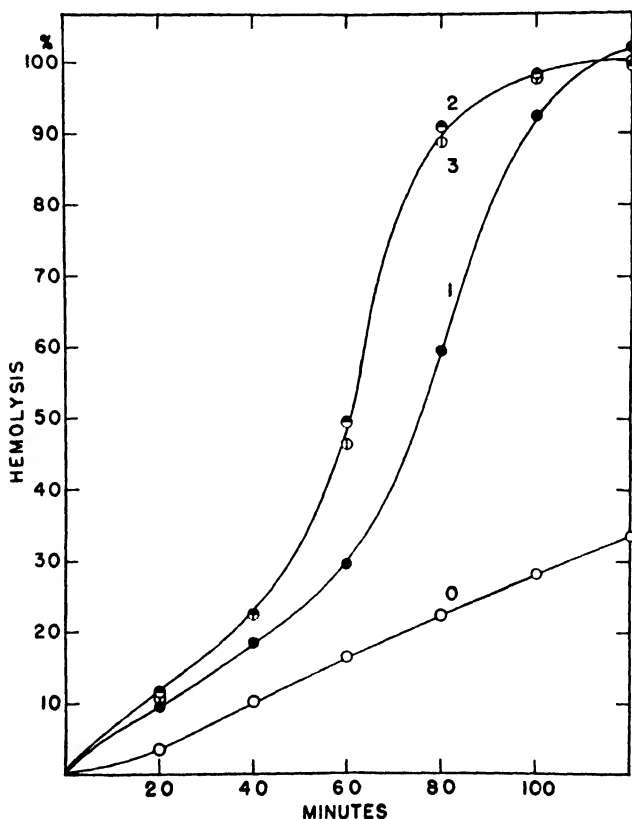
Fig. 1. Effect of the removal of plasma and washing of red cells on the hemolytic activity of gramicidin.

Human blood diluted 1:200 with isotonic NaCl solution. Gramicidin concentration 0.060 mg./100 ml. Curve 0: whole blood. Curve 1: cells washed once with 17 vol. 0.87% NaCl solution; plasma removed together with supernatant wash fluid. Curve 2: cells washed twice. Curve 3: cells washed 3 times.

definition of the antihemolytic agent in plasma, experiments were carried out with washed red cells and the addition of various protein fractions.

The proteins under investigation were dissolved in isotonic NaCl solution and added to an isotonic suspension of red cells. The volumes were adjusted so that 100 ml. of the mixture contained the erythrocytes from 0.5 ml. blood. The hemolytic agents were added 10 min. after the addition of the protein and the hemolysis was followed turbidimetrically at 21–23°C. as described before (1). All hemolytic substances were used in isobacteriostatic concentrations, *i.e.*, in the concentration required to inhibit the growth of *Staph. aureus* (A.T.C.C. No. 6538) by 50% under the conditions reported earlier (1). That the change in the sensitivity of the red cells toward gramicidin is due to the removal of a protective agent and not to mechanical damage caused by washing and centrifugation, is demonstrated in Table III, which shows that

TABLE III

Effect of Human Serum and of Bovine Albumin on the Hemolysis of Washed Human Red Cells by Gramicidin and Gramicidin Derivatives

Hemolytic agent	Concentration γ/100 ml.	Per cent hemolysis at 120 min. in presence of:			
		No protein added	Cryst. bovine albumin, 12.5 mg./100 ml.	Bovine albumin fraction V, 12.5 mg./100 ml.	Human serum 0.25 ml. per 100 ml.
Gramicidin	45	100	99	98	36
Methylol gramicidin	20	17	14	11	1.5
R-46	77	17	8	5	0.3
R-48	80	26	16	9	2

the addition of serum restores the resistance toward the hemolytic substances. Table III also illustrates that albumin is considerably less effective than whole serum.

Fractionation of beef serum with ammonium sulfate, carried out as described previously (4), revealed that the antihemolytic activity was present mainly in the globulin fraction soluble in 0.39 saturated (1.6 M) and insoluble in 0.50 saturated (2.05 M) ammonium sulfate solution. This fraction contained 8.8% of the protein in the starting material. The effect of bovine serum fractions obtained from the Armour Laboratories on the hemolysis of human red cells by gramicidin is illustrated in Fig. 2. The protective action is located in fraction IV-1, which consists (at least in human

plasma) mainly of α-globulins and a considerable quantity of lipide material (5). The slight activities of fractions III-1 and V are probably also due to the presence of small amounts of α-globulins.
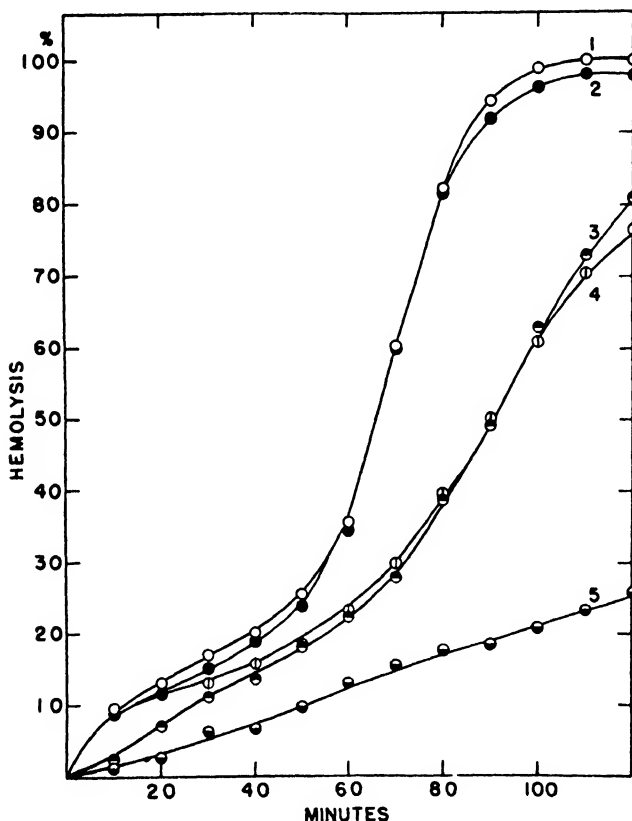
FIG. 2. Effect of bovine serum protein fractions on the hemolytic activity of gramicidin.

Human blood cells, washed 3 times and diluted 1:200 with isotonic NaCl solution. Gramicidin concentration 0.045 mg./100 ml.; protein concentration (when added) 10 mg./100 ml. Curve 1: hemolytic effect of gramicidin, no protein added. Curve 2: serum protein fraction II added. Curve 3: serum protein fraction III-1 added. Curve 4: serum protein fraction V added. Curve 5: serum protein fraction IV-1 added.

C. Effect of Protein on the Antibacterial Activity

It has been stated that proteins interfere with the antibacterial activity of tyrocidine but do not affect the bacteriostatic efficacy of

gramicidin (6). There was reported, however, a mild degree of inhibition by serum, which appeared to be due principally to the cephalins (6).

Experiments to be described in this report led to the conclusion that the bacteriostatic activity of gramicidin is markedly reduced in the presence of certain plasma protein fractions. Bacteriostatic tests were performed as described earlier (1), but to 10 ml. cultures there were added 2 ml. portions of various plasma protein solutions, which had been sterilized by Seitz filtration. Per cent growth values were obtained as usual with the aid of suitable calibration curves and the concentration of the various compounds required for 50% inhibition of bacterial growth was determined as previously described (1). The effects of the presence of 1% crystalline bovine serum albumin on the bacteriostatic activity of gramicidin and its derivatives are summarized in Table IV.

TABLE IV

*Effect of Bovine Serum Protein Fractions on the Antibacterial
Activity of Gramicidin and Gramicidin Derivatives*

·Substance	$\gamma/100$ ml. required for 50% inhibition *Staph. aureus*		
	No protein added	1% Cryst. bovine albumin	0.1% Bovine serum fraction IV-1
Gramicidin	45	20	130
Methylol gramicidin	23	21	190
R-38	133	129	——
R-41	125	121	-
R-42	77	109	360:97% growth
R-46	77	97	——
R-51	76	98	——
R-52	48	82	720:82% growth
R-47	212	280	720:96% growth
R-48	80 .	87	360:71% growth

This protein caused an increase in the activity of gramicidin against *Staph. aureus*. The activity of the formaldehyde derivative (methylol gramicidin) remained unchanged, whereas the other compounds either experienced some loss in potency (R-42, R-46, R-47, R-51, and R-52) ranging from 26 to 41%, or retained practically full activity (R-38, R-41, and R-48).

Experiments with 2 commercial samples of crude bovine serum albumin fraction V showed that the two samples were surprisingly different in their effects on the bacteriostatic activity of gramicidin and its formaldehyde derivative. The first albumin sample (Lot C-1846, concentration 1%) did not alter the activity of gramicidin. The potency of methylol gramicidin was reduced, however, so that 86 instead of 23 γ/100 ml. were required to suppress the growth of *Staph. aureus* by 50%. The second sample (Lot C-2010) not only raised the methylol gramicidin concentration required to give 50% inhibition of growth to 109 γ/100 ml., but interfered markedly with the activity of gramicidin. In the presence of 1% of this crude fraction, 206 instead of 45 γ of gramicidin were needed/100 ml. for a 50% inhibition of growth. These results indicated the presence in crude bovine albumin fraction V of an impurity, varying in amount from one batch to another, and capable of strongly antagonizing the antibacterial activity of gramicidin.

Further experiments showed that the bovine serum fraction II in a concentration of 0.1% had no effect on the antibacterial activity of gramicidin, whereas fraction III-1 in the same concentration raised the gramicidin level required for 50% inhibition of the growth of *Staph. aureus* to 0.063 mg./100 ml., *i.e.*, by about 40%. Fraction IV-1 had the most pronounced effect on the antibacterial activity of gramicidin and its derivatives, as shown in Table IV. It has been pointed out already that fraction IV-1 contains a considerable quantity of lipide material and it is this same fraction which accounts for the inhibitory action of whole serum on the hemolytic activity of gramicidin.

D. Antibacterial Activity of Gramicidin Derivatives in vivo

1. Pneumococcus. Dr. R. J. Dubos found that 0.006 mg./20 g. mouse of compound R-52 (0.3 mg./kg.) were required for 50% protection of mice against 10000 MLD's of *Pneumococcus* (Type I, Strain SV 1), when both the drug and the infective agent were administered intraperitoneally. Under these conditions 12 times as much of compound R-52 is required as of gramicidin to produce this survival rate. On intravenous administration R-52 showed only 1/85 of the toxicity of gramicidin (see Table II).

2. Streptococcus hemolyticus. Dr. Harold J. White carried out a series of experiments to determine the effect of compounds R-47, R-48, and R-51 on infections produced in mice by the intraperitoneal injection of *Streptococcus hemolyticus*, strain 203 C. The *subcutaneous* administration in propylene glycol of the 3 compounds listed, in amounts up to 32 mg./kg., failed to protect the animals. No death occured as a result of the subcutaneous injection of the same amounts of the compounds in healthy control animals. The *intravenous* treatment with 1 mg./kg. of compounds R-48 and R-51 and with 8 mg./kg. of R-47 did not protect the infected animals. *Intraperitoneal* administration of these compounds immediately after infection protected a number of mice as shown in Table V.

TABLE V

Effect of Gramicidin Derivatives and Penicillin on a
Streptococcus Infection in Mice

Organism: *Streptococcus hemolyticus;* Strain C 203. Mice: Tumblebrook Farms; 18–22 g.

Infection: Intraperitoneal; 0.5 ml. of a 10^{-5} dilution of a 5 hour blood broth culture; 4300 organisms.

Treatment: Intraperitoneal; single dose of each drug in 0.2 ml. of propylene glycol given immediately after infection.

Substance	Dose in mg./ kg. mouse	Number of animals alive on 14th day from a total of 10 used for each dose
R-47	8	2
R-47	4	3
R-48	2	4
R-48	1	5
R-51	8	7
R-51	4	7
Penicillin G	4	7
Penicillin G	1	5
Untreated	–	0

E. Chemical Data

A few chemical tests were performed to determine whether there existed any pronounced chemical differences between gramicidin and the gramicidin derivatives. Total nitrogen analyses were carried out by the semimicro Kjeldahl procedure as described by Clark (7). In each case the HI treatment was employed to assure complete digestion of the tryptophan portion of the molecule. Control tests with this amino acid gave theoretical values. The nitrogen content of the various derivatives is listed in Table II.

A modification of the Voisenet-Rhode reaction (8) was used for qualitative trypto-phan tests. The procedure was as follows: about 0.5 mg. of the material to be tested was dissolved in 3.0 ml. of glacial acetic acid, 0.5 ml. of a 5% aqueous solution of p-dimethylaminobenzaldehyde was added, and the mixture brought to a volume of 20 ml. by the addition of concentrated HCl. Upon addition of 0.2 ml. of 1% sodium nitrite solution, the deep blue color characteristic for tryptophan appeared with gramicidin and with compounds R-41, R-42, R-46, and R-48. Methylol gramicidin gave a violet-red color, and compound R-47 gave a blue solution with a greenish cast.

All gramicidin derivatives were tested for newly formed acidic groups by titration of alcoholic solutions with alcoholic KOH, using thymolphthalein as indicator. Most compounds did not react, with the exception of R-47, which neutralized 1 mole of alkali/1200 g., and R-48 which had an apparent neutral equivalent of 3700. Titrations of acetone solutions with alcoholic HCl and naphthyl red as indicator revealed one basic group/3800 g. of R-47. Compounds R-41, R-51, and R-52 neutralized amounts of acid corresponding to one basic group per 3700, 2500, and 2700 g., respectively.

Acetylation experiments were carried out (9) to determine the number of hydroxyl groups in gramicidin and in several gramicidin derivatives. Results with gramicidin, and with methylol gramicidin, were easily reproducible and indicated the presence of 2 and 7 hydroxyl groups per molecule, respectively, in accordance with earlier reports (6, 10). Tests with the new gramicidin derivatives showed rather wide fluctuations. This was apparently due to a more or less incomplete reaction of the substances, which became highly viscous when warmed with acetic anhydride and pyridine. The average results indicated that the chemical treatment had not added any hydroxyl groups to the starting material.

F. Preparation of R-51 and R-52

R-51. To a solution of 1.40 g. gramicidin (0.50 mM) in 250 ml. 95% alcohol, was added 100 ml. of a solution containing 60 mM NH₂OH in 0.12 N NaOH. The mixture was kept for 17 hrs. in a thermostat at 70 ± 0.1°C. (reflux condenser), and the product was then flocculated by the addition of 1 l. of 0.1 N NaCl solution. The precipitate was separated by centrifugation, washed 3 times with 200 ml. portions of water, and dried in a vacuum desiccator over CaCl₂. The crude product (1.145 g.) was extracted twice with 20 ml. portions of 95% alcohol. Evaporation of the combined extracts at 20–25°C. yielded 0.61 g. alcohol-soluble material. The concentration of this substance required to inhibit the growth of *Staph. aureus* by 50% was 0.076 mg./100 ml., and the hemolytic activity at this concentration was 7% of that of an isobacteriostatic concentration of gramicidin. The growth of *Strep. Lancefield* was inhibited 50% at a concentration of 0.107 mg./100 ml.

R-52. This substance was prepared exactly as R-51, except that the temperature was kept at 68 ± 0.1°C. during the 17 hr. reaction period. The yield of alcohol-soluble material was 0.69 g. This substance inhibited the growth of *Staph. aureus* by 50% at a concentration of 0.048 mg./100 ml.; at this same concentration the hemolytic activity was 11% of that of gramicidin. The growth of *Strep. Lancefield* was inhibited 50% at a concentration of 0.082 mg./100 ml.

DISCUSSION

The toxicity tests described in this report show that gramicidin is more effective against mice than against *Staph. aureus*. About 1.3 mg. gramicidin were required/l. medium (containing 0.1% bovine serum protein fraction IV-1) to reduce the growth of *Staph. aureus* by 50%, whereas about one-tenth of this quantity/kg., administered intravenously, proved lethal to mice. Serum protein fraction IV-1 lowered considerably not only the antibacterial effectiveness but also the hemolytic activity of gramicidin. The presence of this protein fraction in human plasma in a concentration of about 5 g./l. (5, 11) makes it impossible safely to inject the amounts of gramicidin required to reach bacteriostatic levels in the circulating blood.

The new gramicidin derivatives considered in this communication have only a small fraction of the toxicity of gramicidin (0.1–1.2%) but retained much (21–94%) of the activity against *Staph. aureus* in regular culture medium. On the surface this looks promising. The addition to this medium of 0.1% of serum protein fraction IV-1, however, interferes with the antibacterial effectiveness of these compounds to such an extent (Table IV) that they have no value for the intravenous treatment of systemic infections. Several of the substances, when administered intraperitoneally, protected mice against intraperitoneal infections with *Pneumococcus* Type I and *Strep. hemolyticus*. The protective dose of R-51 against *Strep. hemolyticus* was about the same as that of Penicillin G (Table V). Compound R-52 compared favorably with gramicidin in its protective action against *Pneumococcus*, considering the ratios between curative and toxic doses for both compounds. These results suggest that some of the new derivatives might be more suitable for the treatment of local infections than gramicidin.

The structural changes which resulted in the low toxicity of the new derivatives in comparison with the starting material are still obscure. Only compounds R-47 and R-48 show a significant decrease in nitrogen content, which is most likely due to hydrolysis (by HCl) and substitution (with iodine), respectively. The presence in R-47 of one acidic group per weight unit of 1200 suggests that this substance has about $\frac{1}{2}$ the size of the gramicidin molecule. Titration with HCl showed only one free amino group (instead of 3) for a molecular weight of 3800, but this is probably due to the formation of a hydrochloride during the preparation of R-47. The complete lack of a free amino

group in R-48 suggests that the acidic properties of this compound are due to oxidative changes rather than to the opening of the peptide ring. This ring was apparently opened in compounds R-41, R-51, and R-52, as indicated by the presence of free basic groups.

Summary

1. Mice were found to be more sensitive to the intravenous administration of propylene glycol than rats. The injection of 5 ml./kg. caused a mortality rate of 20%, and 7 ml./kg. was regularly lethal.

2. The LD_{50} of gramicidin, given intravenously to mice, was determined to be about 0.03 mg./kg., and of methylol gramicidin about 0.36 mg./kg. The new gramicidin derivatives had from 0.1 to 1.2% of the toxicity of the starting material.

3. The addition of small amounts of the bovine serum protein fraction IV-1 reduced markedly the hemolytic and bacteriostatic activities of gramicidin and its derivatives.

4. Compound R-52 (0.3 mg./kg.; intraperitoneal) protected mice against intraperitoneal infection with *Pneumococcus* Type I. Compounds R-47, R-48 and R-51 (1–8 mg./kg.; intraperitoneal) protected a number of mice against intraperitoneal infection with *Strep. hemolyticus*.

5. The chemical data available at present do not permit an explanation for the very high toxicity of gramicidin, nor do they reveal the structural changes which resulted in the comparatively low toxicity of the new gramicidin derivatives.

6. Gramicidin and its derivatives are not considered of value for the intravenous treatment of systemic infections. The results of the toxicity tests and of the intraperitoneal treatment of mice suggest, however, that some of the new derivatives might be more suitable for the treatment of local infections than gramicidin.

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Studies on Wheat Germ Lipase. I. Methods of Estimation, Purification, and General Properties of the Enzyme[1]

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INTRODUCTION

Despite the fact that the existence of plant esterases was definitely established by Green (1) as far back as 1891, there have been very few quantitative studies of the physicochemical properties of these enzymes.

The literature on plant esterases[4] has been reviewed recently (3, 4), and therefore only a few of the salient papers will be mentioned here.

Among the lipolytic enzymes of plant tissues castor bean lipase has been studied most extensively, probably because of its relatively high activity, although, at least in regard to its pH optimum, it seems to be atypical among plant lipases (5). Little attention has been paid to the lipases of cereal grains, although such studies should be of considerable practical importance. Kretovich (6) investigated the lipase activity of wheat in relation to moisture content. In a preliminary paper Sullivan and Howe (7) reported on the lipase activity of various milling fractions of wheat. Using ground, unpurified wheat as a source of enzyme, they found a pH optimum of 7.3–8.2 with triacetin as a substrate under their experimental conditions. The pH optimum with higher trigly-

[4] The terminology and classification of esterases used in this paper is the one adopted by Sumner and Somers (2).

cerides as substrates was on the acid side. They reported that the enzyme preparation acted on a wide variety of simple esters and triglycerides, the activity on triacetin being greatest. The fact that enzyme activity toward the higher triglycerides increased during germination and remained unchanged toward the lower glycerides may indicate that these authors were dealing with a mixture of esterases rather than with a single enzyme. The unusually great differences in pH optima would also point to the same possibility.

Sullivan and Howe tried various methods of extraction but were unable to concentrate the enzyme. Their data on specificity and pH effects are limited by their choice of an assay method. The use of a 24-hour incubation period and subsequent titration of the fatty acid liberated precludes the measurement of *initial* reaction rates, a prerequisite for kinetic data.

Since the purpose of the present investigation was to describe the lipase of wheat germ in terms of the laws of enzyme kinetics, the initial task was to develop methods that would allow rate measurements of lipase action. The first paper in this series deals with the methodology and describes the general properties of the enzyme. The following paper (8) will present the kinetic data obtained.

Experimental

Reagents

The esters used in this study were either commercial preparations purified by high vacuum distillation before use or were synthesized in the laboratory. "Tween 20" and "Tween 40" were products of the Atlas Powder Company. The arsenicals were generously provided by Dr. Harry Eagle, Johns Hopkins University, Baltimore; the *p*-chloromercuribenzoic acid and iodosobenzoic acid were prepared in the laboratory.

Methods

Since previous methods used for the assay of lipases of cereal grains were unsuitable for the quantitative determination of reaction rates, the first problem was to establish methods that would permit the measurement of initial rates of enzyme action, using water-soluble substrates to provide homogeneous systems, and experimental condi-

tions under which the activity of a single enzyme was being measured as the *limiting* factor.

Two methods were adapted for this purpose. The first one of these was based on the manometric determination of carbon dioxide evolved from a bicarbonate buffer, as a result of the liberation of fatty acids by the enzyme. Originally, this method was proposed by Rona and Lasnitski (9) for the estimation of lipases in animal tissues and body fluids, using the poorly soluble substrate, tributyrin. A similar method was used by Nicolai (10) for lipase assay in fir seeds and seedlings, but using mono-*n*-butyrin, a substrate of considerably greater solubility. The second method used in these kinetic studies involved incubation of the enzyme at any desired pH with an ester of one of the lower fatty acids and subsequent deproteinization and steam distillation of the fatty acid liberated.[5] The advantage of this method over the manometric technique resides in the fact that any buffer that is non-volatile with steam could be used and thus the pH range in which experiments could be carried out was not limited to the effective buffering range of the bicarbonate-carbonic acid system. It does not, however, permit the continuous observation of reaction rates and is more time-consuming than the first method. Details of the two methods are given in the following section.

Manometric Method. The method, as routinely used in the course of the present study, was as follows. The enzyme was placed in the main compartment of a conventional Warburg vessel, followed by bicarbonate solution and other additions. The substrate, dissolved or emulsified in a bicarbonate solution of the same molarity as was the solution in the main compartment, was pipetted into the side arm of the vessel. (This prevented the initial gas evolution when the enzyme and substrate were mixed.) There were 0.075 millimols of $NaHCO_3$ in a total volume of 3 ml.; thus the molarity of the bicarbonate was 0.025. In equilibrium with 5% CO_2-95% N_2 gas this gave a pH of 7.4 at 38°C. All assays were made at this temperature, unless otherwise indicated. After gassing for 3 minutes with the above gas mixture, followed by temperature equilibration for 10 minutes, the contents of the side arm were tipped in, and the evolution of CO_2 followed from 3 to 33 minutes. The manometers were shaken at approximately 150 oscillations per minute during this time.

Under these conditions the total CO_2 evolution was strictly proportional to the enzyme concentration, provided the substrate was in sufficient excess. The standardized conditions for activity measure-

[5] After completion of the work the attention of the authors was called to the fact that an entirely similar method of esterase assay had been used by Mattick and Kay (11).

ments during the course of purification, and in much of the kinetic work, employed 1.12×10^{-1} M monobutyrin as substrate, with the system at pH 7.4 and 38°C. Under these conditions there was a linear relationship between enzyme concentration and carbon dioxide evolution for at least 30 minutes, provided the CO_2 evolved did not exceed 200–300 μl. during this period.

The substrate was usually dissolved or emulsified in bicarbonate buffer and then placed in the side arm. With some of the sparingly soluble glycerides, 1% gelatin in 0.025 M NaHCO$_3$ was substituted as a solvent to increase the solubility or to stabilize the emulsion. Substrate concentrations were adjusted so that, after mixing the enzyme and substrate, complete solution of the latter resulted. Particularly in the early stages of purification the proteins of wheat germ enhanced the solubility of the less soluble triglycerides sufficiently to allow the use of excess substrate. Under these conditions the hydrolysis followed a zero order reaction.

In view of the fact that the enzyme was somewhat unstable at the pH of 0.025 M bicarbonate, it was the general practice to keep the enzyme in water or in dilute phosphate buffer at pH 6–7 until the assay was started. The bicarbonate was then added separately.

The enzyme blank was insignificant even in the crudest preparations, but it was necessary to correct for the substrate blank (no enzyme) in each case, because of the small but appreciable hydrolysis of all the substrates tested at pH 7.4. Duplicate or triplicate determinations agreed within 2–3%.

After a single step in purification, the CO_2 retention by the enzyme preparation became negligibly small. In crude water extracts and homogenates, however, retention was significant and it had to be taken into account in the few cases where the activities of crude germ samples were compared. This was done by tipping in 10 μM of HCl from the side arm into a vessel containing the same amount of enzyme and bicarbonate as in the regular assay system, and noting the recovery of CO_2. In the crude preparations there was about 23% retention, and this was constant regardless of the total amount of CO_2 evolved. Much of the retention was due to the presence of dialyzable buffers which were removed in the early stages of purification. Thus, no correction was necessary in the kinetic experiments wherein partly purified preparations were used. In experiments where the pH or temperature were varied, the strength of the bicarbonate was adjusted accordingly, using the Henderson-Hasselbach equation and checking the calculated pH by means of a glass electrode in an atmosphere of 5% carbon dioxide.

Fig. 1 illustrates the proportionality of CO_2 evolution to enzyme concentration and the time course of the reaction, using a water homogenate of defatted wheat germ. It is seen that the enzyme is the limiting factor under these conditions. In experiments of 30–60 mins. duration, zero order reaction was obtained.

Steam Distillation Method. In practice mono-*n*-butyrin was the substrate most commonly used in this method. One ml. of an emulsion containing 10% by volume

of the glyceride was added to 2.0 ml. of 0.05 M phosphate (or other) buffer of the desired pH, followed by enough water to adjust the volume to 6.0 ml. after addition of the enzyme. The contents were brought to 38°C. and the solution of the enzyme was added at zero time. The entire experiment was carried out in test tubes, which were shaken in a Warburg bath at 38°C. for 30–60 mins., depending upon the extent of hydrolysis required. As many as 36 tests could be made simultaneously by starting them at 30 second intervals. At the end of the incubation, the action of the enzyme was stopped by addition of a fresh solution of 10% (by weight) of HPO_3 to give about pH 3. The amount of deproteinizing agent required to give this pH was previously determined on an aliquot of each enzyme preparation. If the pH was much above 3, recovery of the butyric acid was not entirely complete; below pH 3, hydrolysis of the
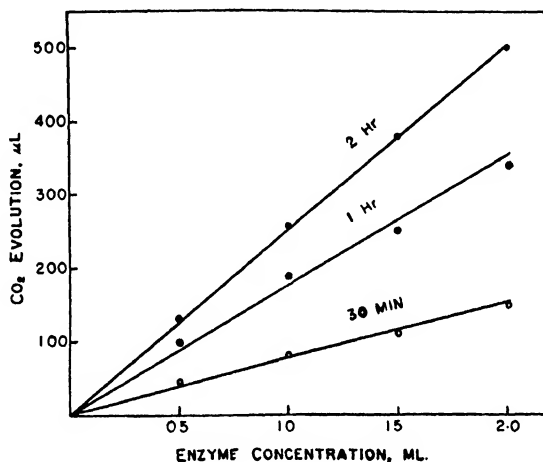
FIG. 1. Relation between enzyme concentration and carbon dioxide liberated during various incubation times (manometric method). One ml. of enzyme preparation contained 40 mg. of homogenized wheat germ. The substrate was 1.1×10^{-1} M monobutyrin.

ester during steam distillation gave rise to appreciable blanks. Following this, water was added to a volume of 12 ml. and the samples were centrifuged for 10 minutes. A 10 ml. aliquot of the supernatant was placed in a Kjeldahl flask and steam distilled at a lively rate until 100 ml. of distillate were collected. The condensate was gassed with N_2 through a sintered glass aeration tube to remove traces of CO_2 and titrated with 0.01 N NaOH with a microburette. The water blank, containing only water and metaphosphoric acid, was less than 0.05 ml. The reagent blank was determined with the complete system, but using boiled enzyme, to correct for the small but significant breakdown of mono-n-butyrin during distillation. The error of the method was 1–2% with 50–100 micromoles titration, and only 3–5% when as little as 20–40 micromoles of butyric acid were determined.

Fig. 2 illustrates a typical experiment showing that the enzyme activity is proportional to the titration under these conditions. The enzyme was prepared by extracting 1 g. of defatted germ with 10 ml. cold water and using the supernatant after 20 mins. centrifugation. For convenience the titration values are converted into μl. (1 micromol $= 22.4$ μl.). As seen in the figure, the enzyme is the limiting factor under these conditions.
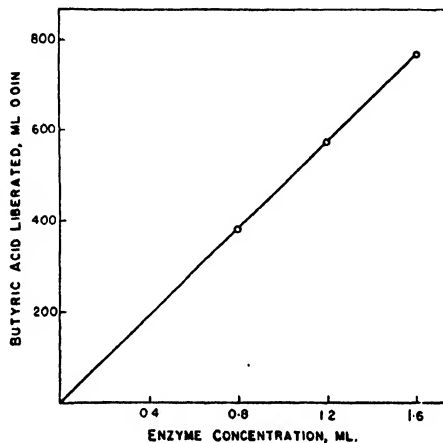
FIG. 2. Relation between enzyme concentration and butyric acid liberated (steam distillation method). Enzyme: aqueous extract, equivalent to 100 mg. wheat germ/ml. Substrate: 1.1×10^{-1} M monobutyrin. Buffer, 2.5×10^{-2} M $NaHCO_3$ -5% CO_2, pH, 7.4. Temp., 38°C. Time, 1 hr. Appropriate blanks subtracted.

Nitrogen was determined by a micro Kjeldahl method using $SeOCl_2$ as catalyst. Protein was determined by a quantitative application of the biuret reaction (12).

Preparation of the Enzyme

The activity of various enzyme preparations was followed by means of the manometric test and expressed as μl. CO_2 liberated/mg. N. In the later stages of the work the biuret method was used to determine the protein content of the solutions.

The source of all enzyme preparations was fresh, untreated wheat germ. The wheat germ was first defatted to remove endogenous fats and to facilitate grinding. Two hundred g. or larger batches of germ were stirred at room temperature at least 5 times with 3 volumes of low-boiling petroleum ether (C.P. or reagent), the solvent

being filtered off between washings on a large Büchner funnel. One hundred g. or smaller lots were more easily defatted in a large Soxhlet apparatus. This caused no inactivation of the enzyme, as this lipase is very stable in the dry state. The dry germ was ground in a Wiley Mill to pass a 40-mesh screen and stored at $-10°C$. The activity of the dry preparation did not change in one year's storage at this temperature. A brief homogenization in a Potter-Elvehjem homogenizer in cold water, or 15 mins. stirring with 10 volumes water, at 3°C. gave apparently·complete extraction. Stirring for 1 hr., or using 0.9% NaCl solution, or 0.154 M NaHCO₃ did not increase the yield of enzyme, although more protein went into solution under these conditions. The enzyme was in true solution since centrifugation for 1 hour at 10,000 r.p.m. left all the activity in the clear supernatant. The latter became turbid on standing in the cold, particularly when bicarbonate was used as the extractant.

The pH of the initial water extracts was 6.6–6.8. This coincides with the pH of optimum stability of the enzyme. In bicarbonate at pH 7.4, or in acetate buffer at pH 5.5, the activity fell fairly rapidly even in the cold. Even at pH 6.6 there was a 10-20% loss in activity in the initial extract on 24 hours storage at 3°C. On the other hand, the enzyme was very stable when stored at -10 to $-15°C$. in the frozen state. Since as many as 20 cycles of freezing and thawing did not affect the activity, stock solutions of the enzyme were preserved at $-15°C$. in the frozen state and thawed just before use.

Glycerol was ineffective in protecting solutions of the enzyme from inactivation. About half of the loss in activity with time was due to $-SH$ oxidation, as evidenced by the fact that in the presence of 0.01 M glutathione and under an atmosphere of nitrogen only 19% inactivation occurred in 3 days at 3°C. as against some 36-40% in the controls.

Dialysis in a rocking dialyzer against running distilled water (3°C.) did not decrease the activity any more than in the undialyzed controls kept at the same temperature. This indicated that no highly dissociated prosthetic group was present in the enzyme. Centrifugation after prolonged dialysis did not precipitate the enzyme; thus its solubility resembled an albumin.

Purification. The purification of the enzyme has not yet been completed and, hence, work along this line will not be reported here in detail. Essentially, two methods were found promising: precipitation with saturated $(NH_4)_2SO_4$ solution in the cold, and acetic acid precipitation at controlled pH's. Acetone and isoelectric precipitation even at 0°C. and in the presence of glycerol or glutathione or glycerides to protect the enzyme, caused considerable losses in activity which nullified any purification achieved by its use. Colloidal adsorbents gave somewhat more promising results. The pronounced heat lability of the enzyme ruled out heat inactivation as a method of removing impurities.

The procedure for a typical preparation was as follows: 150 g. defatted germ powder were extracted by stirring with 1500 ml. water at about 3°C. for 15–30 minutes. The extract was centrifuged for 20 minutes in the cold, and the cloudy super-

natant (1300 ml.) was adjusted with 0.5 N acetic acid at 3°C. to pH 5.5 (1 l. solution required 20 ml. 0.5 N acetic acid). The resulting suspension was centrifuged for 10 minutes (3000 r.p.m.), and the clear solution, containing all of the enzyme, was re-adjusted to pH 6.6–6.8 with 0.5 N NaHCO$_3$ in the cold (1 l. clear solution required 40 ml. of the bicarbonate solution). To the resulting solution, saturated (NH$_4$)$_2$SO$_4$ solution was added slowly with good stirring to 35% saturation, and the precipitate, containing only small amounts of activity, was discarded. To the clear supernatant more (NH$_4$)$_2$SO$_4$ solution was added to bring the saturation to 55%. At this point essentially all the enzyme was precipitated. The precipitate was separated by centrifugation for 30 minutes in the cold and lyophilized. The resulting dry powder retained its activity for at least 11 months in the cold and was also rather stable at room temperature. Before use, it was dissolved in 0.02 M phosphate buffer, pH 6.8 and dialyzed for two hours. Table I compares the activities of the various fractions mentioned above.

TABLE I

Lipase Activity of Various Fractions

Fraction	Activity in mm.^{3}/mg. N	CO$_2$/30 min. /mg. dry weight
Defatted wheat germ homogenized in water	—	2.5
Water extract of defatted germ after centrifugation	35–40	—
After pH 5.5 precipitation	80	—
(NH$_4$)$_2$SO$_4$ precipitate, lyophilized, dialyzed	175	28.0

Manometric assay: 1.12×10^{-1} M monobutyrin, pH 7.4, 38°C.

The experiments reported in this and in the following paper (8) were performed with this type of preparation or with the supernatant obtained after precipitation at pH 5.5. As will be shown later, such preparations seem to contain only one enzyme acting on the substrates used in this work and were, for that reason, deemed adequate for the kinetic experiments.

Specificity

All preparations of wheat germ lipase examined acted on mono- and triglycerides of the lower fatty acids, on simple esters, and on "Tween 20" and "Tween 40" (polyoxyalkylene sorbitan esters of lauric and palmitic acids, respectively).

True glycerides of the higher fatty acids were not used as substrates because of their insolubility and the consequent difficulty arising in the interpretation of kinetic data in heterogenous systems. Since the work of Gomori (13) and of Archibald (14) has shown that the water-soluble

sorbitan esters of the higher fatty acids (Tweens) are suitable substrates for various animal lipases, the hydrolysis of these esters by wheat germ lipase was taken as an indication that wheat germ lipase would act on the higher, as well as the lower, glycerides.

In a recent paper, Glick and Fischer (15) attempted to localize lipase in wheat grains using Gomori's histochemical technique (13) and "Tween 40" as substrate. They failed to find any indication of enzyme action on this substrate in any of the sections. They called attention to the fact that the lipase activity of wheat is of low order and that possibly the particular ester used is hydrolyzed slowly or not at all. Actually, the lipase activity of wheat germ preparations compares favorably with certain pancreatic extracts. In our experience "Tween 40" was consistently, but slowly, split in the manometric test, employing crude or purified germ extracts. The discrepancy in these findings suggests, as would be expected, that germ is richer in this type of lipase than whole wheat grains.

Sullivan and Howe (7) report that ground wheat contains an enzyme for the hydrolysis of the higher triglycerides, although this action was much slower than the splitting of the short-chain glycerides. Their observation, however, may possibly reflect only the relative solubilities of these glycerides, rather than true differences in specificity.

In accord with the observation of Sullivan and Howe it was found that triacetin was attacked by the enzyme more readily than any of the other substrates tested. It may be pointed out that the nonenzymatic hydrolysis of triacetin by the buffer (pH 7.4), was also higher than the blank hydrolysis obtained with any of the other esters. In general, the lability to hydroxyl ions, of all esters examined, as determined in the manometric blanks, seemed to parallel closely the rates of enzymatic hydrolysis, as though the enzyme merely increased the chemical lability of the substrate.

Table II gives a survey of the relative rates of hydrolysis of various substrates by wheat germ lipase. The compounds in Part I of this table were assayed after the corresponding Michaelis-Menten constants had been determined. These values, therefore, represent maximum rates of hydrolysis, as the substrates were present in just sufficient concentration to saturate the enzyme. Inasmuch as the K_m values for the compounds in Part II of the table were unknown, these substrates were arbitrarily tested at a concentration of 1.12×10^{-1} M (enzyme saturation level for monobutyrin). Therefore, these values do not necessarily denote maximum initial activity.

The relative rates of hydrolysis of various substrates may not necessarily reflect the specificity of an enzyme. Even where an enzyme is

TABLE II

Relative Rates of Hydrolysis of Various Esters by Wheat Germ Lipase

Substrate	Concentration of substrate	Relative rate of hydrolysis (1.12×10^{-1} M monobutyrin $= 100$)
Experiment no. 1		
Triacetin	2.5×10^{-1} M	552
Ethylene glycol diacetate	3×10^{-1} M	314
Tripropionin	4×10^{-2} M	200
Methyl acetate	5×10^{-1} M	167
n-Propyl acetate	2×10^{-1} M	144
Monopropionin	2.5×10^{-1} M	134
n-Butyl acetate	1×10^{-1} M	130
Ethyl acetate	4.5×10^{-1} M	115
Monobutyrin	1.12×10^{-1} M	100
Tween 20	5%	89
Tributyrin	2×10^{-2} M	85
Tween 40	5%	35
Experiment no. 2		
Ethyl formate	1.12×10^{-1} M	199
Methyl glycine	1.12×10^{-1} M	48
Ethyl butyrate	1.12×10^{-1} M[a]	16.8
Methyl butyrate	1.12×10^{-1} M	9.6
Methyl propionate	1.12×10^{-1} M	0

[a] Not completely dissolved. Enzyme: $(NH_4)_2SO_4$ precipitated; dialyzed. Manometric assay. Experimental conditions as in Table I. Blank values subtracted.

working at maximum rate on each substrate; that is, where each substrate is present in sufficient excess to saturate, but not yet inhibit, the enzyme, other factors may complicate the interpretation of the results. Thus, it has been pointed out by Longenecker (3) that in instances where there is optical specificity in the hydrolytic action, the maximum initial velocity is the resultant of the hydrolysis of the two optical isomerides present in a racemic mixture. No attempt was made in this investigation to study this point. When long digestion periods are used, with triglycerides as substrates, the observed rate may be the resultant of the saponification of mono-, di-, and triglycerides. Also, the chemical lability of the substrate may play an important role, as appeared to be the case here. It would seem, therefore, that the Michaelis-Menten constants of the enzyme for a series of substrates is a more reliable measure of the specificity. These K_m values for wheat germ lipase were determined and will be presented in the next paper of this series.

The relative rates of hydrolysis of these substrates by wheat germ lipase did not change in the course of purification. This suggested that a single enzyme may be responsible for the activation of the triglycerides, simple esters, as well as of the "Tween" type of high molecular weight esters. Evidence to bear on this point was obtained by partial inactivation of the enzyme by heat, acid, and alkali and subsequent assay on a series of different substrates. If more than one lipase had been present in wheat germ, it would be expected that denaturation of the preparation by different agents would alter the relative rates of hydrolysis of the various substrates. The experiments reported in Table III were designed to test this point. They were performed on

TABLE III

The Effect of Heat, Acid, and Alkaline Inactivation of Wheat Germ Lipase on the Hydrolysis of Various Substrates

Treatment	Substrate used for assay of partially inactivated enzyme	Activity		Inactivation
		Control	Treated	
		$mm.^3$ CO_2		Per cent
Five min. at 48.2°C. at pH of opt. stability (6.6)	Monobutyrin	109.7	92.9	15.3
	"Tween 20"	71.9	61.1	15.1
Fifteen min. at 50°C. at pH 6.6	Monobutyrin	91.7	37.2	59.4
	Ethyl acetate	47.1	16.9	59.8
One hr. at pH 5.5 and 38°C.	Triacetin	227.5	168.8	25.7
	Monobutyrin	74.0	53.0	28.3
	Ethyl acetate	22.5	16.8	25.3
One hr. at pH 8.0 and 38°C.	Triacetin	227.5	189.6	16.7
	Ethyl acetate	74.0	60.5	18.2

centrifuged water extracts of wheat germ and confirmed later, using a more purified fraction. A sample of the enzyme was rapidly brought to the pH or temperature stated, and kept there for the period indicated, using a constant temperature bath. Then they were rapidly cooled in ice and aliquots assayed in triplicate on a series of substrates. In the case of the pH inactivation studies, the amount of acid or alkali required to reach the desired pH was previously determined on an aliquot of the enzyme, to facilitate rapid handling; at the end of the incubation period the enzyme was adjusted to the pH of optimum

stability and assayed immediately. As seen in Table III, all 3 denaturing agents tested gave exactly the same extent of inactivation on substrates as different as ethyl acetate, triacetin, monobutyrin, and "Tween 20," regardless of the extent or method of inactivation. This was interpreted to mean that a single enzyme catalyzes the hydrolysis of all these esters. Further evidence to support this interpretation came from kinetic data. When the maximum rates of the splitting of tributyrin, triacetin, monobutyrin, and ethyl acetate were separately determined, and then two substrates were added to the enzyme preparation (each in a concentration sufficient to saturate the enzyme), the rates of hydrolysis were not additive. The CO_2 evolution in the manometric test lay between the two rates obtained with the two substrates used singly. Where the enzyme had a high affinity (low K_m) for one substrate, like tributyrin, and a low affinity (high K_m) for the other, like ethyl acetate, the rate was very nearly the same as for tributyrin alone. This indicated that the enzyme was saturated with respect to the triglyceride and ethyl acetate could not efficiently displace the other substrate. While absolute proof for the existence of a single enzyme must await the isolation of the enzyme in the homogeneous state, the above experiments strongly suggest that one lipase is responsible for the activation of the various esters used in this work.

Prosthetic Group Requirements, Inhibition, Reactivation

Because of the well-known fact that calcium salts increase the activity of other lipases, such as pancreatic lipase (16) and cottonseed lipase (17), it was of interest to determine whether Ca^{++} might not affect this enzyme in a similar manner. It was mentioned earlier that dialysis did not alter the activity of the enzyme. Addition of 0.02 M $CaCl_2$ solution before or after dialysis, to crude extracts or to the purified enzyme in either the presence or absence of oxalate, did not change the activity. Sodium oxalate at a concentration of 0.01 M did not inhibit, and the activity in 0.016 M phosphate and citrate buffers was the same as in veronal and borate buffers, although the first two of these efficiently bind Ca^{++} and the latter do not (Table IV). NaF, another complexing agent for bivalent cations, had no effect at a concentration of 0.001 M and inhibited only 28% at 0.01 M concentration. This small effect of fluoride ions is unlike the extensive inhibition obtained by F^- at much lower concentrations with enzymes requiring Ca^{++} or Mg^{++} for full activity. Other halide ions did not inhibit the

TABLE IV

The Effect of Various Buffers on Wheat Germ Lipase

Buffer	pH	Incubation time	Activity (ml. of 1×10^{-2} M butyric acid liberated)
		hrs.	
Phosphate	7.8	1	2.22
Veronal	7.8	1	1.85
Borate	7.8	1	1.75
Phosphate	6.5	2	2.89
Citrate	6.5	2	2.87

Conditions: 1.6 ml. enzyme in a total volume of 6.0 ml.; 1.66×10^{-2} M buffers, 1.2×10^{-1} M monobutyrin. Deproteinized with HPO_3 and butyric acid determined by steam distillation.

enzyme at 0.01 M concentration. It might be added that Mg^{++}, Mn^{++}, or heated enzyme extracts were also without effect on the activity of wheat germ lipase. On the basis of these data it may be concluded that Ca^{++} or Mg^{++} are not essential components of this enzyme.

While reagents which form complexes with Ca and Mg ions had no significant effect, all sulfhydryl reagents that were tested inhibited the enzyme at low concentrations. The enzyme was far more sensitive to the action of low concentration of specific sulfhydryl poisons than pancreatic lipase (18), while liver and serum esterases do not require intact —SH groups for full activity.

The action of some typical reagents for the detection of sulfhydryl groups in enzymes is recorded in Table V. The extent of inhibition seemed to depend on the particular substrate used for assay, systematically varying with the molecular dimensions of the latter. An extensive study was made on this phenomenon (19) and the complete data will be published elsewhere.

The data in Table V compare the action of various sulhydryl reagents on wheat germ lipase, using monobutyrin and triacetin as substrates. The best evidence that tying up of the —SH groups of the enzyme decreases or abolishes its activity came from the use of the highly selective mercaptide forming reagents, p-chloromercuribenzoic acid and trivalent organic arsenicals. Sufficiently high concentrations of any of these reagents completely and instantaneously inactivated the enzyme. Ten equivalents of glutathione, added 15 mins. after the poison, effected considerable, though usually incomplete, reactivation. Cysteine was somewhat less effective than glutathione as a reactivator.

TABLE V

The Effect of —SH Reagents on Wheat Germ Lipase

Enzyme preparation	Amount of enzyme/vessel	Substrate	Inhibitor	Inhibition	Reactivator	Reactivation
	mg.			Per cent		Per cent
Crude water extract of germ	11	Monobutyrin, 0.11 M	p-Chloromercuribenzoic acid, 1×10^{-3} M	100	—	—
Crude water extract of germ	11	Triacetin, 0.11 M	p-Chloromercuribenzoic acid, 1×10^{-3} M	87	Glutathione, 1×10^{-2} M	65
Crude water extract of germ	11	Monobutyrin, 0.11 M	p-Aminophenylarsine oxide, 1×10^{-3} M	90	Glutathione, 1×10^{-2} M	33
Crude water extract of germ	11	Triacetin, 0.11 M	Ferricyanide, 2×10^{-3} M	43	—	—
pH 5.5 precipitated	6.1	Monobutyrin, 0.11 M	p-Chloromercuribenzoic acid, 1×10^{-1} M	77	—	—
pH 5.5 precipitated	6.1	Triacetin, 0.25 M	p-Chloromercuribenzoic acid, 1×10^{-1} M	68	—	—
(NH$_4$)$_2$SO$_4$ precipitate	5.4	Monobutyrin, 0.11 M	Iodoacetamide 5×10^{-3} M	55	—	—
(NH$_4$)$_2$SO$_4$ precipitate	5.7	Monobutyrin, 0.11 M	p-Aminophenylarsine oxide, 5×10^{-5} M	62	—	—
(NH$_4$)$_2$SO$_4$ precipitate	5.7	Monobutyrin, 0.11 M	o-Iodosobenzoate 1×10^{-3} M	86	—	—
(NH$_4$)$_2$SO$_4$ precipitate	5.7	Triacetin, 0.25 M	o-Iodosobenzoate 1×10^{-3} M	47	—	—

Manometric assays: total volume, 3.0 ml. Conditions as in Table I.

During the course of purification the enzyme became increasingly more sensitive to the action of —SH poisons.

Summary

A lipase from wheat germ was obtained in soluble form and partly purified. The enzyme acted on a large series of water-soluble simple esters, mono- and tri- glycerides, and on the laurate and palmitate esters of sorbitan derivatives (Tween 20 and 40). Evidence was obtained to show that in all probability a single enzyme is responsible for the activation of these different substrates. The enzyme is a water-soluble protein, inhibited by all reagents commonly used for testing —SH enzymes. Ca^{++} did not affect the activity.

Two assay methods were adapted to the measurement of the kinetics of wheat germ lipase; one involved the manometric measurement of the hydrolysis of esters in a bicarbonate buffer and the other the steam distillation of the fatty acids liberated.

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Studies on Wheat Germ Lipase[1] II. Kinetics

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INTRODUCTION

The first paper in this series described the general properties of a lipase obtained in soluble form from wheat germ (1). The enzyme acts on simple esters, triglycerides, and on the water-soluble long-chain fatty acid esters of sorbitan ("Tween" 20 and 40). Evidence was presented that the same enzyme is probably responsible for the hydrolysis of these various esters, although only partial purification has been achieved to date. Two assay methods were described which appeared suitable for the measurement of initial rates of hydrolysis. One of these involved the manometric measurement of the hydrolysis of esters in bicarbonate buffer, and the other utilized the volatility with steam of the lower fatty acids liberated as a result of enzyme action.

The present communication deals with the kinetic data obtained by the use of these assay methods.

EXPERIMENTAL

Order of Reaction

The enzyme was prepared, and its activity determined, by methods described in the first paper. At the outset, it was ascertained that, under the conditions of the manometric test (excess substrate) a linear relationship was obtained for 60 mins. in crude extracts, when

CO_2 evolution was plotted against time. In somewhat purified preparations the rate began to fall off slightly after 20–30 mins. Addition of more substrate neither restored the rate nor stabilized the enzyme. It was soon found that the decline in rate with time was due to inactivation of the enzyme at 38°C. (temperature optimum under the experimental conditons), rather than to accumulation of the end products of the reaction. Even at 20°C. qualitatively the same phenomenon took place. For these reasons precautions were taken whenever possible, to keep the reaction time within the limits where a constant
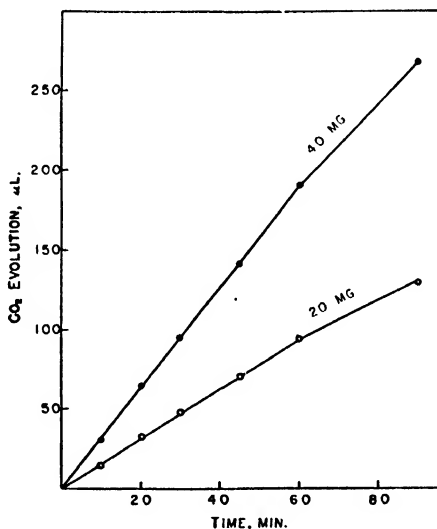
Fig. 1. Time course of the hydrolysis of monobutyrin by wheat germ lipase. Manometric method. Enzyme, defatted germ homogenized in water. Substrate, 1.12×10^{-1} M monobutyrin; buffer, 0.025 M $NaHCO_3$–5% CO_2; vol., 3 ml.; temp., 38°C. Blanks subtracted.

rate of zero order was observed. Under these conditions the rate was strictly proportional to the enzyme concentration. Fig. 1 illustrates the time course of the reaction, using an aqueous homogenate of defatted wheat germ as a source of the enzyme.

The Effect of pH on the Stability of the Enzyme

It has been frequently pointed out in the literature on enzyme kinetics that the so-called "pH curve" is not a true characteristic of

an enzyme, but is a function of two variables: the stability of the enzyme protein to [H⁺] and the effect of [H⁺] on the catalyzed reaction. As a result, the pH optimum may depend on a number of factors, such as substrate concentration, nature of buffer, purity of enzyme, and temperature.

It was deemed necessary, therefore, to study separately the effect of pH on the stability of the enzyme and on the catalyzed reaction. Since the $NaHCO_3$-H_2CO_3 buffer used in the manometric test precludes covering a wide pH range, the steam distillation method was employed. It was mentioned in the previous communication that the enzyme had the same activity in phosphate, citrate, borate, veronal, and bicarbonate buffers; thus, a wide choice of buffers was available for studying the relation of pH to the stability of the enzyme.

Since the apparent "temperature optimum" of the lipase was 38°C., and all assays were performed at that temperature, the pH stability was also studied at 38°C. Since solutions of the enzyme deteriorated fairly rapidly, even at low temperatures (except when frozen), samples previously incubated at 38°C. at various pH values were assayed at pH 6.8 which is close to the stable range of the enzyme. The experiment was performed as follows:

A 16 ml. aliquot of a centrifuged aqueous extract of wheat germ (1 ml. $=o=$ 100 mg. defatted wheat germ powder) was titrated with 0.1 N NaOH from its original pH of 6.7 to pH 10, using a dipping glass electrode and noting the amount of alkali required to reach pH 6, 7, 8, 9, *etc*. Another 16 ml. aliquot was similarly titrated with 0.1 N H_2SO_4 to pH 3, noting the amount of acid needed to reach each intermediate pH interval. Into each of a series of 18 test tubes a 1.6 ml. aliquot of the enzyme was placed, followed by enough 0.1 N H_2SO_4 or 0.1 N NaOH (as determined above) to give the desired pH. Each tube was placed in a water bath at 38°C. immediately after addition of the acid or alkali and shaken continuously for exactly 60 minutes. Each tube was then removed from the water bath and 2 ml. 0.05 M phosphate buffer, pH 6.83 were added, followed by sufficient 0.1 N H_2SO_4 or 0.1 N NaOH to bring the pH back to 6.8, the amounts having been previously determined on larger aliquots. Following this, water was added to give a total volume of 5.0 ml., and the tubes replaced in the water bath. When they reached 38°C., 1.0 ml. of a 0.67 M emulsion of monobutyrin in water was rapidly placed in each experimental tube and also in 2 tubes containing boiled enzyme, which served as blanks. The final volume was thus 6.0 ml. and the molarity of the monobutyrin 0.112; this amount of substrate was sufficient to saturate the enzyme and yet go into true solution. After 2 hours incubation sufficient 5% HPO_3 was added to each tube to lower the pH to 3 and an aliquot of the supernatant after centrifugation was analyzed for butyric acid by steam distillation.

The results of a typical experiment are illustrated in Fig. 2. An almost symmetrical curve was obtained when the pH of incubation (in the absence of substrate) was plotted against the activity. The pH of optimum stability was in the range of 6.6–7.0, that is, at about the original pH of aqueous extracts of wheat germ. As seen in Fig. 2, even at pH 8.0, 38% more destruction occurred in one hour than at pH 6.6. This is undoubtedly the reason why the "pH optimum" of this enzyme is closer to neutrality than that of several plant lipases investigated by Bamann and Ullmann (2) which were most active at pH 8.6–10.5,
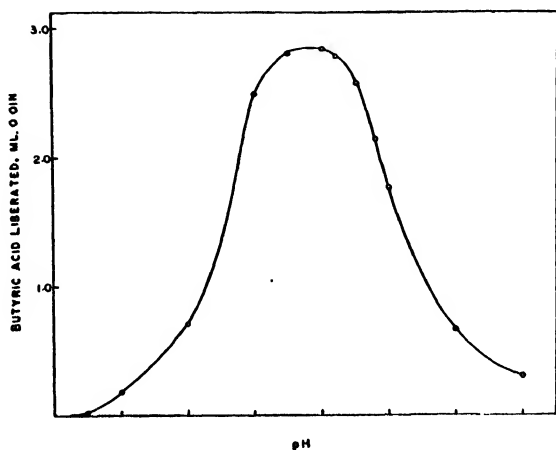
FIG. 2. Effect of pH on the stability of wheat germ lipase in absence of substrate at 38°C. Blanks subtracted. Experimental conditions in text.

the only exception being the lipase of ungerminated cottonseed; the latter acting best at 4.7.

It was important to perform the assay at the pH of optimum stability, since the activity determination entailed incubation for 60–120 mins. during which time appreciable destruction of the enzyme occurred at all pH values, except 6.6–6.8. In that range, 10% or less destruction was found in 2 hours at 38°C. as compared with a frozen control. The pH of optimum stability of an enzyme determined by this method is not necessarily the same as that which would be found if the substrate were present during the acid and alkaline treatment. However, attempts to protect the enzyme with monobutyrin, tributyrin, or triacetin during acid and alkaline inactivation gave negative results.

Thus, it may be assumed that the pH destruction of the enzyme in the presence of substrate, as described in the succeeding section, would be essentially the same as in Fig. 2.

pH Curve

The non-enzymatic hydrolysis of the esters under study was negligible between pH 4–7, under the experimental conditions, but above 7 the saponification by hydroxyl ions rose markedly with the pH. Above pH 9 the breakdown of the substrate by hydroxyl ions became so pronounced as to preclude accurate assays.
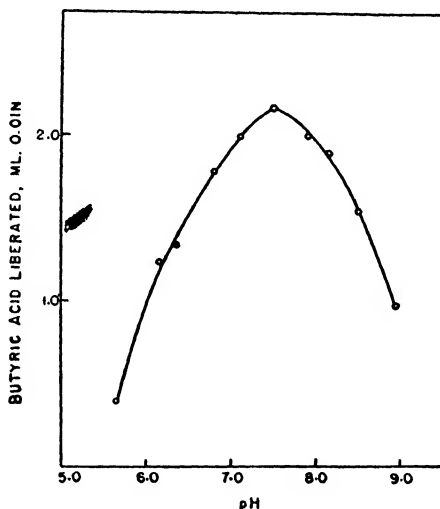
FIG. 3. Effect of pH on the activity of wheat germ lipase. Conditions: 1.6 ml. centrifuged water extract of wheat germ, 1.12×10^{-1} M monobutyrin, 2.84×10^{-2} M PO$_4$ $- 2.84 \times 10^{-2}$ M borate mixtures in 6.0 ml. total volume. Temp., 38°; 1 hr. Butyrate liberated determined by steam distillation. pH values were checked with the Cambridge glass electrode at $t = 0$. All blanks subtracted.

Since the pH activity curve of an enzyme is the net result of changes in pH on the stability of the enzyme and on the speed of the catalyzed reaction, the pH optimum depends on the experimental conditions. Since wheat germ lipase is unstable in the alkaline range which increases the reaction rate, it is to be expected that the pH optimum observed would be nearer pH 7, the longer the experimental period. As shown in Fig. 3, the pH optimum in experiments of 60 mins. dura-

tion was at 7.4–7.5 (steam distillation method). In 20 min. experiments by the manometric method, the optimum was somewhat higher (pH 7.6). The nature of the buffer did not influence the pH activity curve, as would be expected from the fact that the enzyme was equally stable in all the buffers tested. The curve shown in Fig. 3 was obtained in experiments with 1.12×10^{-1} M monobutyrin as substrate; triacetin and ethyl acetate yielded comparable results.

Using ground wheat as a source of enzyme, Sullivan and Howe (3) reported a pH optimum of 7.3–8.2 on triacetin and a much lower optimum on higher triglycerides. While comparison between their values and those obtained in the present study is open to question because of the wide difference in experimental conditions, an optimum range of 7.3–8.2 would be rather unlikely with our lipase preparations in experiments of long duration. There is no assurance that the enzyme mixture measured in the experiments of Sullivan and Howe contained mainly the same lipase, expecially since they stated that the bulk of the activity of their enzyme did not reside in the fractions which were richest in germ.

Effect of Temperature on the Stability of the Enzyme

Reaction Order of Heat Inactivation. The temperature-activity curve of an enzyme, like the pH curve, can be resolved into two components: the effect of increase in temperature on the stability of the enzyme and on the rate of the catalyzed reaction. As characterized by energies of activation (E), the former depends on the experimental conditions, while the latter is usually independent of them, except for reagents which alter the catalyst (4).

The heat stability of an enzyme is best expressed in terms of the energy of inactivation under specified conditions. To calculate this, it was first necessary to determine the reaction order of the heat inactivation of lipase. This was performed as follows:

A 5 g. sample of defatted germ was extracted with 50 ml. water and centrifuged for 20 mins. Twenty-five ml. of this supernatant (pH 6.6) were rapidly brought to 50°C. in a water bath (regulated to ±0.05°C.) and stirred continuously. Five ml. aliquots were removed at 5, 10, 15, and 20 mins. and immediately cooled in a freezing mixture to 10°C. When the last sample was removed, 0.8 ml. portions of the heated enzyme and of an unheated control were assayed by the manometric method with 1.12×10^{-1} M monobutyrin as substrate.

Since the CO_2 evolution in 30 minutes was proportional to the active enzyme concentration, the former was plotted against the time of heating at 50°C. This did not yield a linear relationship, and neither

did a plot of the log CO_2 against time of heating. Hence the heat inactivation was not a zero or first order reaction. When the reciprocal of the CO_2 evolution was plotted against the time of heating, however, a straight-line relationship was obtained, indicating that the heat inactivation of this lipase followed a second order reaction. The relevant data of a typical experiment are summarized in Table I.

TABLE I

Heat Inactivation of Lipase in the Absence of Substrate

Time of heating at 50°C.	Residual enzyme activity as mm.³ CO_2		
	Activity	Log activity	Reciprocal activity
mins.	*mm.³ CO_2*		
0	103.8	2.016	0.0096
5	62.6	1.797	0.0160
10	48.95	1.690	0.0204
15	42.65	1.630	0.0234
20	33.66	1.526	0.0297

Experimental conditions in text.

The finding of a heat inactivation of second order for this enzyme was rather unexpected, as most enzymes follow a first order rate in heat inactivation (4). For this reason, the phenomenon was studied in detail by varying the pH at which the heating took place, as well as the temperature and the time of heating. Under a variety of conditions a second order rate was obtained. An illustration of this is given in Fig. 4, which shows the results of a heat inactivation experiment carried out at pH 7.5 in 1.64×10^{-2} M phosphate-1.64×10^{-2} M borate buffer, at 49°C. for various periods with the same type of enzyme as that used in securing the data in Table I. The steam distillation technique was used for assaying the activity of the heated and control preparations, with 1.12×10^{-1} M monobutyrin as substrate (38°C. 120 mins.). The linear relationship obtained is characteristic of a reaction of second order.

The heat stability of the enzyme was not affected by the presence of any of the substrates studied.

Energy of Inactivation of the Enzyme. The Arrhenius equation predicts that when the log of the specific reaction rate (k) is plotted against the reciprocal of the absolute temperature (T), a straight line

relation should obtain. The slope of this line, multiplied by 4.58 is the energy of activation of the reaction (E). In a second order reaction the dimensions of the specific reaction rate constant are conc.$^{-1}$ × time^{-1}, concentration being expressed in terms of molarity. Since the enzyme
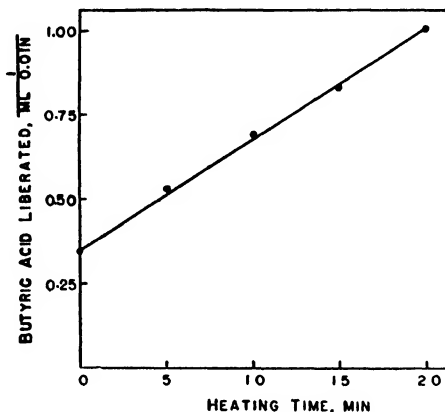
FIG. 4. Inactivation of lipase as a second order process. Abscissa, minutes of heating of the enzyme in the absence of substrate at 49°C. and pH 7.5. Ordinate, reciprocal of the butyrate liberated in subsequent assay in ml. 0.01 N NaOH, as determined by steam distillation and titration. The butyrate titer represents the amount of undestroyed enzyme. Conditions in text.

preparation used was impure and the molecular weight of the enzyme was unknown, the following relation was used to express the specific reaction rate (k)

$$k = \frac{[\text{Inactive enzyme}]}{[\text{Active enzyme}][\text{Total enzyme}][\text{Time}]}.$$

The ratio of inactive to active enzyme was experimentally determined, and the total enzyme concentration was arbitrarily taken as 100. This was permissible when log k was plotted against $\frac{1}{T}$. Since the total enzyme concentration and the time of heating were constant, the value chosen for total enzyme concentration did not change the slope of the curve. The slope thus obtained, multiplied by 4.58, gave the energy of inactivation (E) of the lipase under the experimental conditions (Fig. 5.)

When the Arrhenius equation was written in the form

$$\ln \frac{k_2}{k_1} = \frac{E}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right)$$

or in the present case

$$\ln \frac{\left[\dfrac{\text{Inactive}}{\text{Active}}\right]_2}{\left[\dfrac{\text{Inactive}}{\text{Active}}\right]_1} = \frac{E}{R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right)$$

and the left side of the equation was plotted against $\left(\dfrac{1}{T_1} - \dfrac{1}{T_2}\right)$, the E obtained was the same as by the above method.
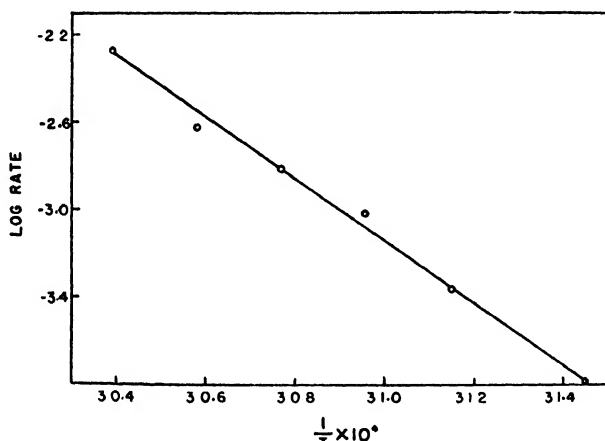
Fig. 5. The effect of temperature on the heat destruction of lipase at pH 6.8. Energy of activation from slope = 65,100 cal./mole. The ordinate represents the log of the specific reaction rate (second order) of the destruction of the enzyme. Conditions in text.

The heat inactivation data represented in Fig. 5 were obtained by rapidly bringing 5 ml. aliquots of a water extract of wheat germ at pH 6.8 to various temperatures ranging from 45° to 56°C. and keeping it at those temperatures for 15 minutes. The tubes were then cooled in ice-salt to 10°C., and an 0.8 ml. sample was analyzed in each case by the manometric method. The activity of the heated samples (triplicates) was compared with an unheated control and the reaction rate and E were calculated from the per cent inactivation, as explained above. At the pH of optimum stability of the enzyme (6.8), the energy of activation for the heat destruction of this enzyme is 65,100 cal./

mole. It is apparent from Fig. 5 that the Arrhenius equation holds for the entire range of temperatures investigated.

To determine the effect of pH on the energy of inactivation of the enzyme, this experiment was repeated at pH 7.5 in 0.0164 M phosphate–0.0164 M borate buffer. After 15 mins. heating at temperatures between 45°–51°C., the samples were cooled and assayed at pH 6.8 by the steam distillation method. The log of the rate of inactivation thus observed was plotted against $\frac{1}{T}$, and the slope of the line gave an $E = 56,000$ cal./mole. Thus, the energy of inactivation was lower at pH 7.5 than at pH 6.8. This was to be expected, of course, from the pH-stability relations of the enzyme. The E values obtained compare favorably with other enzymes (4), and are higher than the value reported for pancreatic lipase at pH 6 (46,000).

Since the molar concentration of the enzyme was unknown, and thus the exact value of k (specific reaction rate constant) for a second order reaction could not be determined, calculation of the entropy of inactivation from the Eyring equation had to be omitted.[4]

Energy of Activation of the Catalyzed Reaction

Having determined the effect of temperature on the stability of the enzyme protein, the next problem was to measure the energy of activation of the hydrolysis of various substrates by the lipase, under conditions where the enzyme is stable. This was most conveniently performed by the manometric method, using a centrifuged water extract of defatted germ powder. The substrates were present in sufficient

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[4] When the Eyring equation

$$k' = \exp. (\Delta S^*/R) \exp. (-\Delta H^*/RT) \cdot \frac{kT}{h},$$

where $\Delta H^* = E^* - RT$, is written in the form

$$\Delta S^* = 1.98 \left[\frac{\log k'}{\log (2.07 \times 10^{10} T)} - 1 + \frac{E}{1.98T} \right],$$

a minimum value of ΔS^* can be calculated by neglecting the uncertain term $\frac{\log k'}{\log (2.07 \times 10^{10} T)}$, which is very small compared to the rest of the equation.

This value is of the order of 200 cal./degree/mole. An entropy of this magnitude and a moderate E value would make reversible heat inactivation improbable. Actually, a variety of experiments designed to reverse the heat inactivation of lipase gave negative results.

concentration to saturate the enzyme. The pH was kept at 7.4 at each temperature by varying the concentration of $NaHCO_3$ in the HCO_3^- — CO_2 buffer, as calculated from the Henderson-Hasselbach equation and the solubility of CO_2 at the given temperature. Since it has been shown that a zero order reaction takes place under the conditions of the manometric test, the corresponding rates were calculated from the formula:

$$k = \frac{C_1 - C_0}{t_1 - t_0},$$

which in terms of CO_2 evolution may be written as

$$k(\text{in min.}^{-1}) = \frac{\mu l.\ CO_2}{22.4} \times \frac{1000\ cc.}{3\ cc.} \times \frac{1}{30} \times 10^{-6}.$$

When the log of the reaction rate was plotted against the reciprocal of the absolute temperature, as in Fig. 6, a straight line was obtained up to 37.9°C., showing that the Arrhenius equation was strictly obeyed both in the case of monobutyrin and of triacetin. Above 38°C. there
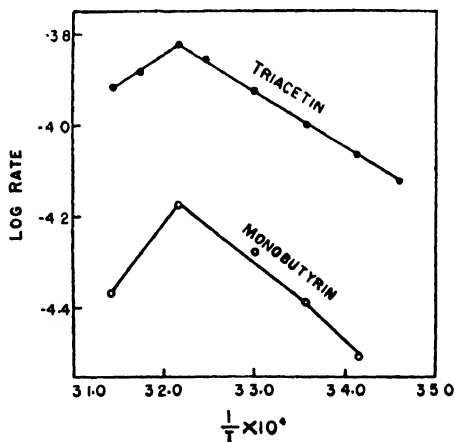
Fig. 6. The effect of temperature on the hydrolysis of some esters as catalyzed by lipase. The ordinate represents the log of the reaction rate for the hydrolysis of the two esters (zero order). E for triacetin = 5,710 cal./mole; for monobutyrin, $E = 7,850$ cal./mole. In the experiments with triacetin 0.5 ml. of a water extract of defatted germ, with monobutyrin 0.8 ml. of a similar preparation, were used. Inflection in curves represents the point where inactivation of the enzyme begins. Experimental conditions in text.

was a sharp break in the curve, coinciding with the point where denaturation of the enzyme became apparent. From the slopes of the lines in Fig. 6, the corresponding activation energies were calculated. For monobutyrin the slope was -712 and hence $E = 7850$ cal./mole. For triacetin, with a slope of $-1,248$, $E = 5,710$ cal./mole.

The energy of activation of the hydrolysis of monobutyrin obtained here agrees quite well with data in the literature for the hydrolysis of tributyrin: 7600 and 8700 cal./mole between 0–50°C., obtained by means of pancreatic lipase (5, 6). Since the same acid and alcohol residues exist in monobutyrin as in tributyrin, the same E value would be expected. This again demonstrates that the energy of activation of the catalyzed reaction is constant, regardless of the nature of the catalyst, under conditions where the latter is stable.
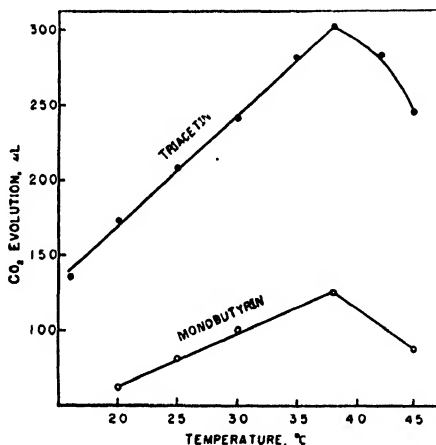
FIG. 7. Relation between temperature and activity of wheat germ lipase. Experimental conditions in text. Blank values subtracted.

Apparent Temperature Optimum

Fig. 7 is a plot of the observed reaction rate at various temperatures, under the standard conditions of the manometric test (pH 7.4, 1.12 $\times 10^{-1}$ M monobutyrin or triacetin). Different enzyme preparations were used with the two substrates, but the results are recorded together for the sake of convenience.

As would be expected from Fig. 6, the apparent temperature optimum fell at about 38°C. with both substrates. Since the enzyme was

stable up to this temperature for short periods of time, a linear increase in the rate was manifest, but at 38°C. and higher temperatures the rate fell off sharply since denaturation of the enzyme exceeded the increase in the rate of the catalyzed reaction. The sharp and sudden break in the curve was, of course, to be expected in view of the great discrepancy in the energies of activation of the two opposing processes: inactivation of the catalyst and increase in the rate of the catalyzed reaction. Similar curves were obtained also with tributyrin and by the use of an $(NH_4)_2SO_4$-purified enzyme. The apparent temperature optimum would probably be different, however, with more highly purified enzyme or at other pH values.

Michaelis-Menten Constants

The relative rates of hydrolysis of a series of substrates by an enzyme do not necessarily describe the specificity of the enzyme. A more reliable picture of the substrate specificity of an enzyme may be gained by the dissociation constants of the enzyme for a series of substrates (Michaelis-Menten constant, K_m). The K_m values were, therefore, determined for a series of substrates, using both a dialyzed acetate-precipitated extract of wheat germ and a purified preparation of the lipase prepared by treatment with $(NH_4)_2SO_4$.

There were several inherent difficulties in attempting to determine the Michaelis-Menten constants of this lipase. First, some of the esters, like methyl and ethyl acetate, are highly volatile, and when used at low concentrations, the gassing procedure with 5% CO_2 had to be carried out at low temperatures and at a minimal gas pressure, to avoid evaporation of the substrate during the gassing process in the manometric test. Second, the reaction period had to be quite short when low concentrations of triglycerides were used as substrates; otherwise the mono- and diglycerides formed in the course of hydrolysis would have been attacked, thereby supplying "extra substrate." The Lineweaver-Burk method (7) of plotting the experimental results is particularly affected by this consideration. Third, some of the glycerides used had a limited solubility in the $HCO_3^- - CO_2$ buffer. In a plot of enzyme activity against substrate concentration or against the negative log of the substrate concentration (pS), saturation of the solution with respect to the substrate might be mistaken for saturation of the enzyme (V_{max}). The Lineweaver-Burk method, however, helps to overcome this difficulty. In these experiments advantage was taken of the increased solubility of various esters in protein solutions and a solution of gelatin or of heat inactivated wheat-germ extract was added to increase the solubility of the less soluble substrates. It had been previously established that neither of these substances affected the activity of the enzyme.

The results of a large number of experiments are summarized in Table II. The experimental values were determined by the use of the standard manometric method, with a 20–30 min. experimental period, and 0.5 ml. enzyme. In arriving at the constants, 3 different methods were used: the CO_2 evolution was plotted against substrate concentration or against the negative log of the substrate concentration and then the reciprocal of the rate was plotted against the reciprocal substrate concentration, corresponding to the last 3 columns of Table II. With some of the substrates, these methods of calculation gave somewhat different K_m values. This is the result of the difficulties mentioned above. In each case, therefore, the value considered most reliable is marked with an asterisk.

TABLE II

Michaelis-Menten Constants of Wheat Germ Lipase

Substrate	K_m		
	From activity substrate concentration plot	From activity -pS plot	From Lineweaver-Burk plot
	M	M	M
Tripropionin	0.0070	—	0.0072*
Triacetin	0.011	0.0096*	0.011
Monobutyrin	0.031	0.029	0.028*
Monopropionin	0.050	0.051	0.046*
Methyl acetate	0.094*	—	0.14
Ethyl acetate	0.090*	0.094	0.14
n-Propyl acetate	—	—	0.036
Ethylene glycol diacetate	0.020	0.021	0.022*

Most reliable value in each series marked (*). For experimental conditions see text.

Thus, high concentrations of n-propyl acetate inhibited the enzyme and no plateau was obtained when maximum activity was reached making difficult the calculation of half-maximum activity. The Lineweaver-Burk method was not affected by the inhibition at high substrate concentrations. Where such considerations did not come into play, good agreement was found with these different calculations (*cf.* monobutyrin).

The most interesting feature of Table II is the marked regularity observed in the K_m values with increasing chain length. In general, the longer the fatty acid or alcohol chain in the same series, or the more the

alcoholic OH groups that were esterified, the smaller was the K_m value; hence the greater was association between enzyme and substrate. Thus, the K_m value of monopropionin (0.046 M) exceeds the value for monobutyrin (0.028 M); the values for methyl acetate (0.094 M) and ethyl acetate (0.090 M) were much greater than those for propyl acetate (0.036 M). Unfortunately, this tendency of the enzyme could not be checked with the natural glycerides of long-chain fatty acids because of the unsuitability of these in either of the present assay methods.

SUMMARY

A study was made of the kinetics of wheat germ lipase. In the presence of excess substrate consisting of either glycerides or simple esters, the enzyme followed a zero order reaction. At 38°C. the pH of optimum stability of the enzyme was approximately 6.8, and the apparent pH optimum was at 7.4 under the experimental conditions. The inactivation of the enzyme by heat followed a second order reaction, and the energies of inactivation were found to be 65,100 cal./ mole at pH 6.8 and 56,000 cal./mole at pH 7.5. The energies of activation of the enzymatic hydrolysis of triacetin and monobutyrin were found to be 7850 cal./mole for monobutyrin and 5710 cal./mole for triacetin. Under the experimental condition the apparent temperature optimum was 38°C. for a series of substrates.

The dissociation constants of 8 of the common substrates were studied and a marked correlation was observed between molecular size and the dissociation constant.

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The Use of a Hog Kidney Conjugase in the Assay of Plant Materials for Folic Acid[1]

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INTRODUCTION

The microbiological determination of folic acid is made difficult by the variable responses of test microorganisms to the different chemical forms of the vitamin which are known to occur in nature (1, 2, 3, 4). The possible occurrence of the various forms in combination with protein (5, 6) and of other precursors of the vitamin (7) may further complicate the assay. Cheldelin *et al.* (8) first used taka-diastase to liberate the vitamin and found it superior to several other enzymes for treating unheated samples. Luckey *et al.* (5) found that taka-diastase released more folic acid from many food stuffs than did acid and alkaline hydrolysis, treatment with a folic acid conjugase, or autolysis. Olson *et al.* (9) reported similar results, but they found that a hog kidney conjugase was more effective than the taka-diastase in treating plant samples. The conjugase was prepared by the method of Bird *et al.* (10) who had found its use with several natural materials gave results in good agreement, except in the case of certain plant extracts, with those obtained by chick assay. Later, Bird *et al.* (11) reported that the results obtained by the microbiological assay of several conjugate preparations, following treatment with the hog kidney enzyme, compared well with those obtained by spectrophotometric determinations of the vitamin. The studies discussed here were made to determine the suitability of the hog kidney conjugase for liberating folic acid from plant samples, and to compare it with other enzymes in this respect.

Although chicken pancreas also contains a highly active folic acid conjugase (12, 13), hog kidneys were chosen as the source of our enzyme because they were readily available and because the conjugase preparations from them were easy to make and store. In this paper,

evidence is presented which indicates that hog kidney conjugase completely liberates folic acid from its conjugate in plant samples, and that certain other enzymes are ineffective in liberating the vitamin.

<div align="center">EXPERIMENTAL</div>

Enzymatic Treatment of Samples and Assay of Extracts for Folic Acid

The general method for the enzymatic treatment of samples prior to microbiological assay follows: To a known weight of sample in an 18 mm. test tube, 5 ml. of 0.1 M phosphate buffer (pH 7.0), or 5 ml. 1% sodium acetate buffer (pH 4.5), or 5 ml. water was added and, unless otherwise noted, the mixture was heated for 3 mins. in a boiling water bath. Addition of the enzyme was then made, and the mixture was incubated under toluene at 37°C. for 24 hrs., heated in a boiling water bath for 2 mins., cooled, neutralized to pH 6.8–7.0, and made up to a convenient volume. Aliquots of the solution were used for the microbiological assay after filtering. Where two enzymes were used in the treatment of the samples, two 24 hour incubation periods were used, one for each enzyme. After incubation with the first enzyme, the mixture was heated in a boiling water bath for 3 mins. and then cooled. When necessary, the pH was adjusted before adding the second enzyme. Samples for the determination of the folic acid content of the enzymes were prepared in all experiments, and were treated in the same manner as were the plant samples. Details of the various treatments (weight of sample, etc.) appear in the tables.

The conjugase used in these studies was a clarified water-extract of hog kidney prepared and stored frozen as described by Bird et al. (10). It was used essentially as described by these authors. Unless otherwise indicated, all other enzymes were commercial preparations.

The extracts, prepared as just described, were assayed for folic acid by one or more of 3 methods, namely, the turbidimetric method of Luckey, Briggs and Elvehjem (14) with *Streptococcus faecalis* (modified to contain salts B in the medium); the turbidimetric method of Roberts and Snell (15) with *Lactobacillus casei* (modified by increasing the period of incubation to 36 hours); and the acidimetric method of Teply and Elvehjem (16) with *Lactobacillus casei* (modified to omit the norit-treated peptone from the medium). Synthetic pteroylglutamic acid (Lederle) was used as a standard in all assays. Enzyme blanks were determined in all cases.

<div align="center">

Comparison of Methods of Assay

</div>

The 3 microbiological methods listed for assay of folic acid were compared by the analysis of several extracts of samples treated with hog kidney conjugase. The results are given in Table I. The data obtained using the turbidimetric and acidimetric methods with *L. casei* show good agreement between the two types of assay, except in

TABLE I

Microbiological Determination of Folic Acid by Various Methods

Samples, as indicated, were incubated with 5 ml. of pH 4.5 sodium acetate buffer and 2 ml. hog kidney conjugase preparation. Preparation of extracts and microbiological assays have been described earlier in this paper. All values have been corrected for the folic acid content of the enzyme.

Sample assayed	Folic acid content (γ/g.)		
	S. faecalis (Turbidity)	*L. casei* (Turbidity)	*L. casei* (Acidity)
1 g. Canned spinach A	0.93	0.84	0.82
1 g. Canned spinach B	0.43	0.41	0.40
0.1 g. Ground barley	0.63	0.51	0.47
0.1 g. Wheat germ	3.3	2.8	2.6
0.2 g. Ground corn	0.23	0.22	0.17
0.2 g. Corn cereal	0.09	0.09	0.05
0.1 g. Timothy hay	3.0	2.3	2.2
0.1 g. Alfalfa hay	3.2	2.3	2.2

the corn samples, where the high starch content of the extracts, even after filtration, may have caused high results with the turbidimetric method. Since this difficulty was not a factor in subsequent studies, the more convenient turbidimetric method was used. The results for *S. faecalis* were higher in almost all cases than were those for *L. casei;* this is typical of a large number of samples which we have assayed with both microorganisms. The reason for this difference in response of the two microorganisms to the various extracts is not known, but the different times of incubations may be the cause. In many studies results of assays with both microorganisms are reported.

Hog Kidney Conjugase

The presence of conjugase inhibitors in certain natural substances, reported by Bird and his coworkers (11, 17), may be responsible for low assay results when hog kidney conjugase is used in digesting samples. The low folic acid content of the conjugase preparation makes it possible to add it in relatively large amounts to many samples; large amounts of conjugase should overcome the effect of the inhibitors. The results obtained in the recovery studies shown in Table II indicate that, if inhibitors occurred in the samples used, their effect was over-

TABLE II

Recovery Studies with Folic Acid Conjugate and Hog Kidney Conjugase

Digestion mixtures: 0.5 g. of the plant samples, or 0.05 g. of the desiccated liver, suspended in 5 ml. pH 4.5 sodium acetate buffer and heated 3 mins. in a boiling water bath; 1 ml. of a solution containing crystalline vitamin B_c conjugate (Parke, Davis and Company) as indicated; 2 ml. hog kidney conjugase as indicated. The method of incubation and preparation of samples for assay has already been described. Enzyme blanks are subtracted, where necessary, in the values given.

Material assayed	γ folic acid (*S. faecalis*)		
	Spinach	New Zealand spinach	Desiccated liver
Sample only	1.35	0.41	0.71
Sample plus 2 γ PGA [a] added as PGA conjugate	3.46	2.44	
Sample plus 2.5 γ PGA added as PGA conjugate			3.18
PGA recovered from the conjugate	2.11	2.03	2.47
Per cent recovery	· 106	101	99

[a] PGA, pteroylglutamic acid.

come by the amount of enzyme employed. Bird *et al.* (10) suggested that conjugase inhibitors might account for the low results obtained by microbiological assay as compared to chick assay for certain plant extracts, but our results indicate that some other explanation is necessary.

Yeast extract contains hog kidney conjugase inhibitor and also high concentrations of folic acid conjugate (10, 11). It was, therefore, used to determine at what levels the conjugase must be added to overcome the effects of the inhibitor when an incubation time of 20 hrs. (37°C.) is used for the enzyme treatment. The data in Table III indicate that 1 ml. of the hog kidney conjugase preparation gives a large enough excess of the enzyme to overcome all of the inhibitor present in the yeast extract. In a similar study (Table IV) it was found that liberation of folic acid from a sample of yeast decreased only when samples were so large that the viscosity of the mixture made uniform addition of the conjugase solution difficult. These results indicated that the use of 2 ml. of the hog kidney preparation with 0.5 g. samples of fresh plant material or with 0.1 g. samples of dry plant material was sufficient to

TABLE III

*Effect of Varying Concentration of Conjugase on the
Liberation of Folic Acid from Yeast Extract*

Digestion mixtures: 100 mg. yeast extract suspended in 5 ml. pH 4.5 buffer, hog kidney conjugase as indicated, and water to make total volume to 15 ml. After incubating at 37°C. for 20 hrs. under toluene, the mixtures were prepared for assay as described earlier in this paper.

Hog kidney preparation used /100 mg. yeast extract (ml.)	Folic acid content of yeast extract [a] (γ/g.)	
	S. faecalis	L. casei
0.00	0.6	6[b]
0.01	0.8	6[b]
0.10	26	40[b]
1.00	75	46
6.00	74	46
10.00	77	48

[a] Enzyme blanks subtracted.
[b] Approximations only possible because of "drifts."

liberate folic acid completely from its conjugate, even in the presence of inhibitors.

The release of folic acid from a few natural materials by the hog kidney conjugase and by a desiccated chicken pancreas preparation

TABLE IV

The Use of Hog Kidney Conjugase in the Assay of Dried Yeast

Digestion mixtures: The weight of dried yeast indicated was suspended in 5 ml. pH 4.5 buffer and autoclaved for 5 mins. at 15 lb., and conjugase preparation was added as indicated. Samples were incubated and prepared for analysis as previously described.

Weight of yeast sample g.	Ml. conjugase preparation used	Folic acid [a] content of yeast (γ/g.) L. casei
0.1	0	1.3
1.0	2	6.2
0.2	2	8.6
0.1	2	8.8
0.05	2	8.4
0.025	2	8.6

[a] Enzyme blank subtracted.

(18) is compared in Table V. Under the conditions of the experiment, the two conjugase sources appeared equally effective in liberating the vitamin. No studies have been made with the partially purified chicken pancreas of Mims and Laskowski (13).

TABLE V

Comparison of Hog Kidney Conjugase and Desiccated Chicken
Pancreas in the Liberation of Folic Acid

0.2 g. ground oats, 0.1 g. desiccated liver or 0.1 g. dried yeast autoclaved in 5 ml. of the buffer indicated. After adding either 2 ml. hog kidney conjugase or 25 mg. desiccated chicken pancreas to samples, the mixtures were incubated and prepared for analysis as previously decribed.

Enzyme used	Buffer used (pH)	Folic acid content of samples (γ/g.) [a] *(S. faecalis)*		
		Ground oats	Desiccated liver	Dried yeast
None	4.5	0.19	10	0.41
Hog kidney conjugase	4.5	0.55	13	9.6
Desiccated chicken pancreas	7.0	0.53	12	8.4

[a] Enzyme blanks subtracted.

Studies with Other Enzymes

Bird *et al.* (10) observed that the conjugase activity of taka-diastase is very low. Hence, it appeared probable, in view of the reports of other workers (5, 8, 9), that the enzyme could liberate folic acid from "bound" forms other than the conjugate. Schweigert and Pearson (6) found the digestion of blood by taka-diastase resulted in a several-fold increase in its folic acid content and postulated that the vitamin in blood was largely bound to proteins. In view of these observations we tried taka-diastase and certain other proteolytic enzymes, alone and in combination with hog kidney conjugase, in an effort to increase the assay values of certain fresh vegetables. The results indicated that the proteolytic enzymes were of no value, either when used alone or when used with the conjugase (Table VI). In most cases they apparently destroyed the vitamin. Taka-diastase caused no apparent destruction of the vitamin, but neither was there any significant increase in the folic acid content of samples digested with it. In further experiments (Table VII) it was found that, in heated plant samples, taka-diastase liberated no folic acid, and that, in unheated samples, it was only slightly more effective than autolysis at pH 4.5.

Digestion of heated taka-diastase with hog kidney conjugase revealed that taka-diastase contains about 2.5 γ/g. of folic acid, about 90% of which is present as the conjugate. The usual method of determining

TABLE VI

Liberation of Folic Acid by Various Enzymatic Treatments

The general method of enzymatic treatment of samples is described earlier in this paper. In this experiment, 0.5 g. samples of fresh plant material ground with a Nixtamal mill were used. The amounts of the various enzymes used were as follows: Hog kidney conjugase, 2 ml.; other enzymes, 25 mg. Where two enzymes were used in treating the samples, two 24 hour digestion periods were used, as described before. The pH of incubation of the samples with the various enzymes was as follows: Hog kidney conjugase and taka-diastase, pH 4.5; pancreatin, Rhozyme P-11 and Protease 15, pH 7.0.

Enzymes used or treatment	Folic acid content (γ/g.) [b]						
	Spinach		Lettuce		Cauli-flower	Lettuce	
	S. F.[a]	L. C.[a]	S. F.	L. C.	S. F.	S. F.[a]	L. C.
Autolysis, pH 4.5			0.11	0.24	0.26	0.50	0.25
Taka-diastase	0.73	1.10	0.10	0.38	0.26	0.50	0.30
Pancreatin	0.32	0.83					
Rhozyme P-11 [c]					0.15		
Protease 15 [c]					0.17		
Hog kidney conjugase	1.10	1.20	0.50	0.60	0.87	0.83	0.76
Taka-diastase, then hog kidney conjugase	1.20	1.30	0.52	0.60	0.95	0.77	0.82
Hog kidney conjugase, then taka-diastase			0.52	0.68			
Pancreatin, then hog kidney conjugase	0.89	1.10					
Rhozyme P-11, then hog kidney conjugase					0.39		
Protease 15, then hog kidney conjugase					0.52		

[a] Samples not heated prior to first enzymatic treatment. S. F., *S. faecalis*; L. C., *L. casei*.

[b] Enzyme blanks subtracted.

[c] Commercial enzyme preparations with proteolytic activity (Rohm and Haas Company).

the enzyme blank for taka-diastase does not measure the conjugate. Therefore, when unheated plant samples are digested with this enzyme, small increases in the folic acid content of the mixture may result from the liberation of the vitamin from the conjugate in the enzyme. Our

TABLE VII

*The Liberation of Folic Acid from Heated and Unheated Plant Samples by
Taka-diastase and by Hog Kidney Conjugase (pH 4.5)*

| Treatment of samples[d] | Folic acid content of samples[a] (γ/g., wet basis) S. faecalis | | | |
	Lettuce (1 g. sample)	Chard (1 g. sample)	Endive (1 g. sample)	Spinach (0.5 g. sample)
Heated samples: [b]				
Incubated, pH 4.5	0.18	0.27	0.17	0.18
Taka-diastase (25 mg.)	0.19	0.25	0.18	0.18
Hog kidney conjugase (2 ml.)	1.40	0.80	0.90	
Unheated samples:				
Autolyzed in 5 ml. water	0.41	0.50	0.46	
Incubated, pH 4.5	0.39	0.61	0.37	0.78
Taka-diastase (25 mg.)	0.51	0.60	0.35	0.95
	(0.45) [c]	(0.54) [c]	(0.29) [c]	(0.83) [c]
Hog kidney conjugase (2 ml.)	1.40	0.79	0.87	

[a] Enzyme blanks subtracted.

[b] Samples heated for 3 mins. in boiling water bath after buffer addition.

[c] Corrected for conjugate content of the taka-diastase (see text).

[d] All mixtures, containing 5 ml. of buffer or water, as indicated, were incubated and prepared for analysis as already described.

studies, and the work of Bird *et al.* (10), indicate that folic acid conjugase is of widespread occurrence in nature. When the values for the unheated plant samples (Table VII) are corrected for the conjugate content of the enzyme, the differences in the results obtained with taka-diastase and with autolysis at pH 4.5 are well within experimental error.

DISCUSSION

Among the various treatments employed for liberating folic acid from its conjugates, digestion with hog kidney conjugase prior to microbiological assay appears to be the most satisfactory. Destroying the enzymes present in the fresh plant tissues by a preliminary heat treatment insures against destruction of the vitamin during the digestion period, and against the release of other "bound" forms of folic acid in the enzyme preparation which may not be released in

incubating the enzyme alone. In view of evidence for the occurrence of unknown precursors of folic acid in rat livers (7), and indirect evidence for the inability of the hog kidney conjugase to completely liberate folic acid from fresh plant materials (19) or plant extracts (10), it is felt that the use of conjugase preparations does not always make it possible to determine the total folic acid content of natural materials. Nevertheless, such conjugases are the most efficient agents known now for liberating the vitamin from plant samples.

SUMMARY

Evidence is presented to show that a hog kidney conjugase preparation, when used in excess, completely liberates folic acid from its conjugates in the plant materials studied. Certain proteolytic enzymes and taka-diastase are of doubtful value in liberating the vitamin from plant tissues.

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Acute Oral Toxicity of Gossypol and Cottonseed Pigment Glands for Rats, Mice, Rabbits, and Guinea Pigs

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INTRODUCTION

It has recently been reported (1, 2) that cottonseed pigment glands, which are separated in an essentially unaltered condition from cotton-seed kernels by a flotation process (3, 4), retard growth of chicks and laying by hens when the cottonseed glands or products containing them are included in the diet of chicks and laying hens.

Gossypol, a polyphenolic yellow pigment, is the principal component, and gossypurpurin, a polyphenolic purple pigment derived from gossy-pol, is the most abundant secondary component found in the pigment glands of cottonseed (5). On the basis of the early work of Withers and Carruth (6), the toxicity of uncooked cottonseed to some animals has been attributed to gossypol. However, it has recently been reported (2) that the addition of cottonseed pigment glands to the diet of chicks produced marked retardation in growth and high incidence of deaths, whereas addition of an equivalent amount of pure gossypol to the diet caused little retardation in growth. No correlation was apparent between the nutritive values for chicks of various cottonseed products and their relative contents of gossypol and gossypurpurin.

The results reported here extend the aforementioned observations to animals other than chickens and provide additional information on

the nature, as well as possible methods for the removal or inactivation, of the toxic component of cottonseed pigment glands.

METHODS

The three samples of cottonseed pigment glands used in the investigation were prepared by the gland flotation process (3, 4) from a single lot of prime cottonseed which was stored in a silo for about 6 months before processing. The first sample was a composite of pigment glands prepared from seed processed between March 8 and April 16, 1946; the second sample was obtained from seed processed between May 16 and May 25, 1946; and the third sample was obtained from seed processed between November 18 and December 11, 1946. The separated glands were stored at 3.3°C. (38°F.) in sealed containers prior to administration.

To determine the effect of heat on the toxicity of pigment glands, samples were placed in covered aluminum pans and heated at 105°C. for 1 hr. To determine the effect of heat in the presence of water on their toxicity, the samples were thoroughly wetted with distilled water and then heated at 105°C. in covered aluminum containers for 1 hr. The samples were then dried in a vacuum desiccator over anhydrous calcium sulfate and ground to a fine powder. To determine the effect of extraction on their toxicity, the glands were extracted with organic solvents by suspending them in relatively large volumes of the solvent (500 ml. of solvent to 200 g. of glands) and allowing the suspensions to stand overnight at 3.3°C. (38°F.). The insoluble residues were then washed by decantation until the supernatant liquid was practically colorless.

Three independently prepared samples of gossypol were tested. The first sample of gossypol was prepared as recently described (7) by alkaline extraction of diethyl ether extracts of flaked cottonseed meats. The other two samples of gossypol were prepared (8) from diethyl ether extracts of cottonseed pigment glands. The gossypol prepared by hydrolyzing gossypol-acetic acid obtained by treatment of ethereal extracts of cottonseed pigment glands with glacial acetic acid was recrystallized from a mixture of diethyl ether and light petroleum naphtha.[2] All of the samples of gossypol were identical with respect to melting point, elementary composition, ultraviolet and visible absorption spectra, and the absorption spectra of their reaction products with antimony trichloride (9). The absorption spectra of the gossypol samples and their reaction products with antimony trichloride were also identical with those observed for gossypol as it occurs in chloroform extracts of the pigment glands (5). The samples of gossypol were stored at 3.3°C. (38°F.) in brown glass bottles prior to administration.

The gossypol content of the pigment glands was determined by the antimony trichloride spectrophotometric method (10) using chloroform solutions prepared from aqueous ethanol extracts (5) of the glands. The content of gossypurpurin in the pigment glands was estimated on the basis of the specific extinction coefficients at 570 mμ of chloroform extracts of the glands prepared as previously described (11).

The acute oral toxicity of cottonseed pigment glands was determined on rats, mice, guinea pigs, and rabbits, while that of gossypol was determined on rats and guinea

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[2] Pentane-hexane mixture, boiling range 95°–130°F.

pigs. All of the preparations were administered by stomach tube in the form of aqueous suspensions. The animals were fasted for 16–18 hours before administration of the suspension, after which they were permitted free access to water and stock diet. The animals were maintained under observation for a period of 7 days or until prior death. At first, all of the animals which died were autopsied, but this practice was abandoned as the number of deaths increased, and uniformity of the pathological conditions was established. The LD_{50} values were calculated by the method of Reed and Muench (12).

RESULTS

LD_{50} values for rats, mice, guinea pigs, and rabbits to which 3 different preparations of cottonseed pigment glands were administered are shown in Table I. The lesser sensitivity of rats and greater sensitiv-

TABLE I

Toxicity of Cottonseed Pigment Glands[a] in Terms of LD_{50} Values

	Rats	Mice	Rabbits	Guinea Pigs
No of animals used				
Sample no. 1	171	43	10	34
2	252	22	4	5
3	92	—	—	—
LD_{50} value, mg./kg.				
Sample no. 1	925	500	350	280
2	1060	950	ca. 600	300
3	1350	—	—	—

[a] The gossypol contents of the gland samples 1–3 were 40.0, 37.6, and 33.7%, respectively; and of gossypurpurin 0.95, 1.15, and 3.15%, respectively.

ity of rabbits and guinea pigs to cottonseed pigment glands is consistent with their relative sensitivities to petroleum naphtha-extracted cottonseed previously reported by Withers and Carruth (6).

Autopsy of the dead animals revealed consistent hyperemia of the gastrointestinal tract, hemorrhagic intestines, renal and splenic congestion, and stoppage of the heart in diastole. These observations are similar to those reported by Withers and Carruth (6) for animals which died after administration of ethereal extracts of cottonseed.

The toxicity of the 3 samples of pigment glands (Table I) decreased with increased time of storage of the seed from which they were prepared. However, the toxicities of the glands were not proportional to

their contents of gossypol, and their toxicities decreased with increasing contents of gossypurpurin in the glands.

Further evidence that gossypol is not the principal toxic component of the pigment glands was obtained by administration of 3 independently prepared samples of gossypol. Administration of the first sample in doses as high as 600 mg./kg. body weight to a total of 40 rats, and in doses as high as 400 mg./kg. body weight to 5 guinea pigs, not only failed to kill the animals, but had no readily observable effect on them. The quantities of gossypol administered were greater than the amounts which were present in the pigment glands which resulted in death in the case of rats and guinea pigs (Table I). The second sample of gossypol was administered to a total of 20 rats in doses as high as 1600 mg./kg. body weight. The larger doses produced the loss in weight and the severe and almost instantaneous diarrhea which nearly always followed administration of pigment glands, but it did not produce death. The third sample of gossypol, administered to a total of 36 rats in doses as high as 5000 mg./kg. was lethal. On the basis of the foregoing results the LD_{50} value of gossypol was calculated to be 3000 mg./kg. body weight for rats by the method of Reed and Muench (12), which value is greatly in excess of that observed with any of the samples of cottonseed pigment glands (Table I).

Samples of the pigment glands were subjected to dry and wet heat and to exhaustive extraction with acetone and ethanol in order to simulate the extremes of conditions to which they would be subjected during processing of cottonseed in the hydraulic- and screw-press, and solvent-extraction methods. As may be seen from the LD_{50} values reported in Table II, heating of the glands in the absence of water had practically no effect on their toxicity, whereas heating them in the presence of water reduced their toxicity to about one-half that of the original glands. Exhaustive extraction of pigment glands with ethanol reduced their toxicity to less than half of their original value, whereas exhaustive extraction with acetone appeared to have removed all of the toxic material from the glands.

DISCUSSION

The toxicity of cottonseed pigment glands for fasting rats, mice, guinea pigs, and rabbits, in contrast to the relatively slight effect which resulted after administration of comparable quantities of gossypol, is

TABLE II

Effect of Different Treatments on the Toxicity to Rats of Cottonseed
Pigments Glands in terms of LD$_{50}$ Values

Sample no.	Treatment of pigment glands	LD$_{50}$	No. of animals used
		mg./kg.	
2	None	1060	252
2	Heated dry for 1 hr. at 105°C.	1110	25
2	Heated dry for 1 hr. at 105°C., powdered	930	25
2	Wetted, heated for 1 hr. at 105°C., powdered	2400	59
3	None	1350	92
3	Heated dry for 1 hr. at 105°C.	1500	62
3	Wetted, heated for 1 hr. at 105°C., powdered	2120	30
3	Exhaustively extracted with ethanol	3200	59
3	Exhaustively extracted with acetone	>6000	58

consistent with the reported (2) effect of pigment glands and gossypol in the diet of chicks. Also, as previously reported for chicks (2), there is no apparent correlation between the toxicity of different samples of pigment glands and their relative contents of either gossypol or gossypurpurin. Therefore, although the possibility of synergistic action of the two pigments has not been eliminated, the present evidence indicates that some component of cottonseed pigment glands other than, or in addition to, gossypol and gossypururin is responsible for the toxicity of pigment glands in the case of chickens, rats, mice, guinea pigs, and rabbits.

The variation in the toxicity of cottonseed pigment glands which is produced by different methods of processing the glands affords some insight into the nature of the toxic component. The observation that exposure to dry heat for one hour did not affect the toxicity of the pigment glands indicates that the toxic principle is thermally relatively stable in the intact glands. The reduction in toxicity which followed heating of the moistened glands suggests that either the toxic material is soluble in water and is unstable in solution at elevated temperatures, or it reacts with water or water-soluble components of the glands which are released under these conditions.

The detoxication (13, 14, 15) of cottonseed meal during processing

by the hydraulic-press method in which relatively large amounts of water are added prior to cooking of the seed, or supplementary cooking of the meal after pressing, has been attributed to detoxication of gossypol through formation of *bound gossypol*, a hypothetical compound of gossypol and protein. On the basis of the present observation, detoxication of the meal by wet heating appears to be attributable to inactivation of the toxic component of the pigment glands rather than to any reaction of gossypol. Since it has also been reported (11) that addition of water during the cooking of cottonseed prior to pressing improves the color of the expressed oil, it would appear that wet-cooking of cottonseed would be advantageous as a standard procedure during processing of cottonseed.

Since all samples of the toxic pigment glands were prepared by floating them on mixtures of commercial hexane and tetrachloroethylene (4), it is evident that neither of these solvents removes appreciable quantities of the toxic component from pigment glands. Previous reports (2, 6) of the toxicity of light petroleum naphtha-extracted cottonseed and of the high nutritive value of diethyl ether-extracted cottonseed present evidence that the toxic material of the pigment glands is not removed by light petroleum naphtha, but is removed by diethyl ether. On the basis of the present results, the toxic material appears to be removed more readily by acetone than by ethanol. Thus, it may be concluded that the toxic material is readily soluble in acetone and diethyl ether and somewhat less soluble in ethanol. Since it has been shown (3, 16) that solvents such as the petroleum naphthas and chlorinated hydrocarbons are incapable of rupturing the heavy walls enclosing the gland pigments, further experiments using ruptured pigment glands are required to determine the effective solubility of the toxic component of the glands in these solvents.

The fact that the pigment glands were only partially detoxified by heating them for one hour at 105°C. in the presence of relatively large amounts of water may account in part for the reported (2) inferiority of hydraulic-pressed meal to diethyl ether-extracted and gland-free cottonseed meals.

ACKNOWLEDGMENT

SUMMARY

1. Oral administration of cottonseed pigment glands in relatively large doses produces death of rats, mice, guinea pigs, and rabbits. Of these species, rats are the least sensitive, and guinea pigs and rabbits are the most sensitive to the pigment glands.

2. The toxicity of cottonseed pigment glands is attributable to some component or components of the glands other than, or in addition to, gossypol and gossypurpurin.

3. The toxic material of cottonseed pigment glands is thermally relatively stable, but it is partially inactivated by the combined action of heat and water. It is not extracted from the glands by petroleum naphthas and tetrachloroethylene but is partially extracted by ethanol, and is completely extracted by diethyl ether and acetone.

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A Serum Polysaccharide in Tuberculosis and Carcinoma[1]

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INTRODUCTION

It has been found (1) that the polysaccharide content of serum varies considerably from the normal in certain diseases. An attempt has been made in this study to determine more of the nature of this polysaccharide, and, from such information, to find a simple method for its evaluation, adaptable for routine clinical analyses.

Previous studies (1) showed that the polysaccharide content of serum, as determined by the carbazole reaction, increased from a normal mean of 103 mg.-% to a mean of 159 mg.-% in far advanced tuberculosis, and to a mean of 145 mg.-% in carcinoma of several types, whereas, in some other diseases, no such increases occurred. It was suggested that, in diseases accompanied by tissue destruction or pathological activity, an increase may occur in serum polysaccharide. In addition to tuberculosis and carcinoma (1, 2) increases have been found in pneumonia (3, 4) and toxemic pregnancy (5). Mayer (6) reported also, in carcinoma cases, an increase in serum mucoid of about 300% above normal, and this fraction was believed to be responsible for a "polarographic carcinoma reaction."

EXPERIMENTAL

Relationship of Polysaccharide to the Serum Protein

In cases where the serum polysaccharide was found to be increased (1), the α_2-globulin, as determined by electrophoresis, was also increased. The question naturally arose as to whether the polysaccharide might be present as an integral part of the α_2-globulin protein, or as a separate component traveling with the same mobility as this protein. An attempt was made to answer this question by analysis. The analyses were carried out by the carbazole method (1, 7) on a group of

isolated normal plasma fractions generously supplied by Drs. E. J. Cohn and Laurence Strong.[2] The properties of the fractions and methods of isolating many of them have been described (8). Table I

TABLE I

Carbohydrate Content of Isolated Plasma Fractions

Fraction	Chief components present	Polysaccharide content on dry weight basis *per cent*
I	Fibrinogen (60%)	1.17
I-2b	Fibrinogen (95%)	1.00
II-1	γ (95%)	1.21
II-2	γ (95%) ·	1.55*
III-0	β_1-globulin (whole fraction)	1.34
III-0	β_1-lipoprotein (95%)	1.57
III-0-5	· β_1-lipoprotein (65%)	2.18
Prep. a	β_1-globulin (lipide-poor)	2.65*
IV-1	α_1(50%) + lipides	3.39
IV-1, Iw	α_1(90%) + lipides	1.64*
IV-4	α $+\beta$	3.73
IV-5	α ·	3.65
IV-6	α_2(95%)	5.83*
IV-9	α_2 $+ \beta_1$	3.26
IV-7 + 8	Albumin $+ \beta_1$	1.50
IV-8	Albumin	0.84
V	Albumin (97%)	0.30
V-crystd. 5 ×	Albumin (99+%)	0.13*

The information concerning the components contained in the different fractions was kindly given by Dr. Laurence Strong.

gives the results of the carbohydrate analyses on these plasma fractions. The fractions of highest purity, the analyses on which were utilized in the calculations shown in Table II, have been starred. The electrophoretic diagrams of these fractions are also shown in Fig. 1. It is clear from Table I that the fraction IV-6-2, which consists of 95% α_2-globulin, had the highest (5.4%) content of polysaccharide. Albumin (fraction 5V crystalline) was lowest in polysaccharide (0.13%).

It is known that an electrophoretic component of serum may repre-

TABLE II

Polysaccharide Content of Sera

Type of sera	Average g.-% found by electrophoresis						Average mg.-% polysaccharide calculated in						Average total mg.-%		
	Average total	Albu-min	α1	α2	β	γ	Albu-mn	α1	α2	β	γ		Found by direct analysis	Calcu-lated as indicated	Diffe-ence
				Globulins					Globulins						
Fetal	*g.-%* 6.44	3.66	0.37	0.57	0.52	1.32	4.76	6.07	33.2	13.8	20.5		81	78.3	2.8
Normal	7.29	3.88	0.58	0.76	1.01	1.05	5.04	9.51	44.3	26.8	16.3		103	101.9	1.1
Tuberculous 81	7.27	3.62	0.56	0.78	1.00	1.30	4.71	9.18	45.5	26.5	20.2		109	106.1	2.9
Tuberculous 82	7.67	3.32	0.64	0.92	1.15	1.65	4.31	10.50	53.6	30.5	25.6		136	124.5	11.5
Tuberculous 83	7.63	2.69	0.78	1.21	1.19	1.76	3.50	12.79	70.5	31.5	27.3		158	145.6	12.4
Tuberculous 84	7.17	3.71	0.59	0.81	1.00	1.06	4.82	9.68	47.2	26.5	16.4		109	104.6	4.3
Tuberculous 85	7.20	3.54	0.62	0.84	1.11	1.08	4.60	10.17	49.0	29.4	16.7		112	109.9	2.1
Sarcoidosis	7.88	3.10	0.61	0.85	1.26	2.06	4.03	10.10	49.6	33.4	31.9		128	128.9	−0.9
Carcinoma	6.66	2.80	0.66	1.09	1.09	1.03	3.64	10.82	58.6	28.9	16.0		145	118.0	27.0
Per cent Polysaccharide used for calcula-tion		0.13 V	1.64 IV-1-W	5.83 IV-6-2	2.65 β1	1.55 II-2									

sent more than one protein and may even contain non-protein sub-
stances. However, it is probable that a high percentage of any serum
electrophoretic component consists of the protein similar to the
isolated pure fraction with that mobility. With the realization of
the possible errors involved, a calculation was made of the total con-
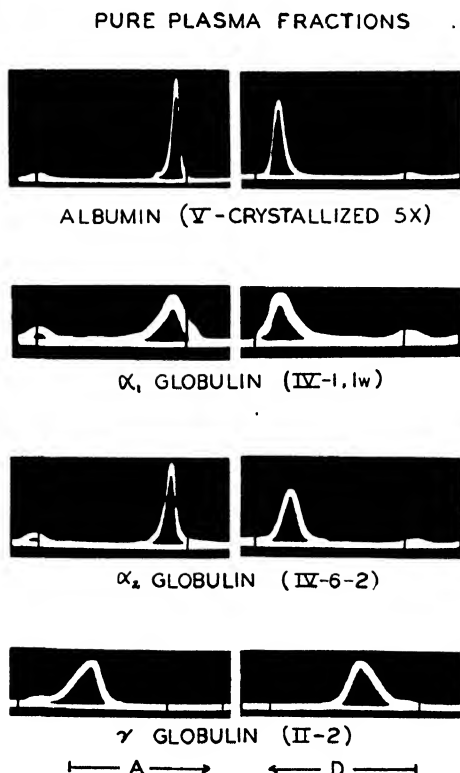
FIG. 1. Electrophoresis curves of purified plasma
fractions, in veronal buffer pH 8.6, $\mu = 0.1$.

tent of polysaccharide of serum, by using the percentages of polysac-
charide found in the respective pure isolated plasma protein fractions
(starred values in Table I), and from these calculating the amount of
polysaccharide in the individual mean electrophoretic components
found in different diseases reported in Table II of an earlier paper (1).
The results, given in Table II in this paper, show that the polysac-

charide values so calculated corresponded well (within -0.9 to $+4.3$ mg.-%) with the mean polysaccharide values found by direct analyses on all the types of sera studied except those from cases of moderately and far advanced tuberculosis and carcinoma. The great discrepancies in these pathological sera suggested the presence of a constituent whose reaction product with carbazole gave a higher degree of absorption than the polysaccharides accompanying the normal protein components. However, in those cases showing the greatest discrepancies between total polysaccharide calculated and that actually found, there was a considerable increase in the polysaccharide accompanying the α_2-globulin fractions. On the other hand, when a considerable increase occurred only in the polysaccharide accompanying γ-globulin, as in the sarcoidosis group, there was no marked discrepancy in calculated and found total polysaccharide.

Nature of Polysaccharide in Serum

It seemed clear, therefore, that the significant polysaccharide for our consideration was the one accompanying the α_2-globulin, and, since this component has been postulated to be increased in conditions characterized by tissue destruction or pathological activity, it appeared possible that this polysaccharide is of a nucleic acid nature. Accordingly, methods of analysis for nucleic acid were applied.

The diphenylamine reaction (9) was tried and a blue color developed, but the method proved unsatisfactory because a precipitate occurred, and also because acid controls alone gave a similar color. In this connection it is to be noted that Stacey *et al.* (10) showed that any substance capable of giving ω-hydroxylevulinic aldehyde under the conditions used in the Dische reaction will give the blue color with diphenylamine.

The method of Seymour Cohen (11), in which the carbohydrates are condensed with tryptophan in the presence of perchloric acid, has proved to be highly sensitive and satisfactory for the development of color with sera. Since only a negligible amount of color is produced with this reagent by free glucose, mannose, galactose, and glucosamine, one determination serves to give the content of the unknown polysaccharide in the serum, and a separate glucose determination, as required with the carbazole reaction (7), is not necessary. Furthermore, this method is far less liable to error than the carbazole reaction. It is a simple test and can, therefore, be highly recommended for routine clinical use.

Cohen (11) has recommended the test as a specific reaction for the carbohydrate of desoxyribonucleic acid, and gives a list of other carbohydrates which show the reaction to varying degrees. Chief among these is fructose. In order to select a proper standard, spectral absorption curves were made with the Beckman spectrophoto-

meter of the tryptophan-perchloric acid reaction mixtures of pure thymus nucleic acid, a digest of thymus nucleic acid,[3] pure ribonucleic acid, adenosine, guanosine, fructose, fructose-6-phosphate,[4] Ba 6-phosphogluconic acid,[4] Ba diphosphoketogluconate,[4] and the calcium salt of 2-keto-gluconic acid.[4] These were compared with the spectral curves obtained on normal and pathological sera (see Figs. 2, 3, and 4).
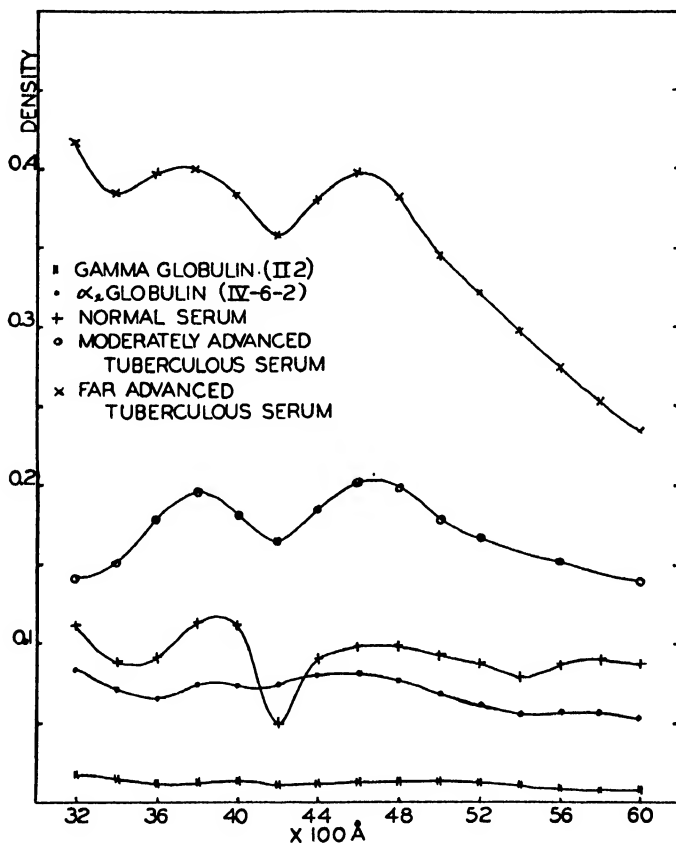
Fig. 2. Spectral absorption curves of perchloric acid-tryptophan condensates of sera and plasma fractions.

The curves showed maxima at 3800–3900 Å and 4600 Å in all normal and pathological sera, as well as for the pure isolated α_2-globulin

[3] This enzymatic digest was prepared from thymus nucleic acid for us by Dr. Charles A. Zittle, to whom we express our sincere appreciation.

[4] These compounds were generously supplied by Dr. Seymour Cohen, and appreciation is expressed for his helpful suggestions.

fraction. The maxima for desoxyribonucleic acid, as well as for the digest mixture, were at 3600 Å and 5000 Å, while the maxima for the ribose nucleic acid and its derivatives, ribose, guanosine and adenosine, were at 3700–3800 Å and at 4500 Å. In none of these substances did
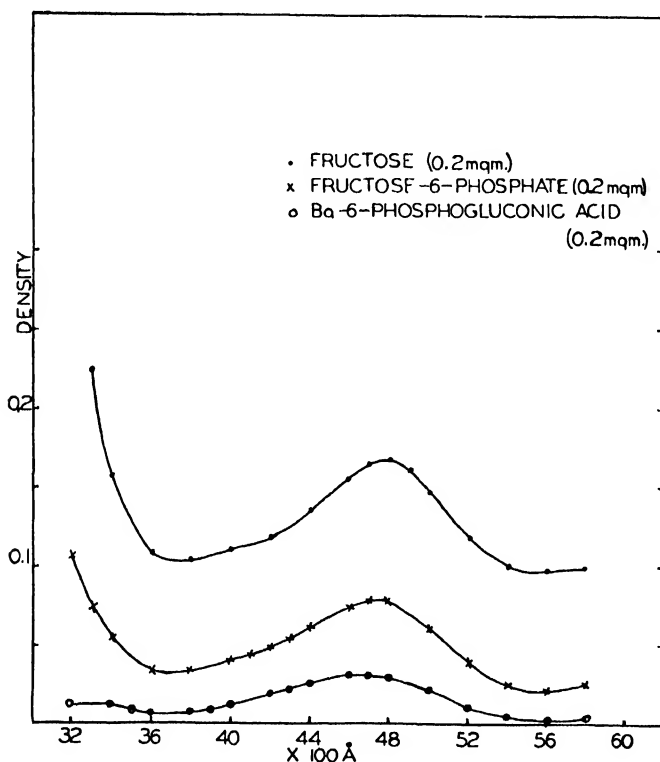
FIG. 3. Spectral absorption curves of perchloric acid-tryptophan condensates of fructose and fructose derivatives.

the absorption correspond exactly with the 4600 Å absorption of the substance in serum. Moreover, the actual color produced in the trypto-phane-perchloric acid condensation of serum was more gray or brown than that produced from these compounds, which is a pale pink, and not completely extractable from solution by isoamyl alcohol, as is the pink color from the nucleic acid. Furthermore, if the substance is a ribonucleic acid compound or derivative, quantitatively there would

have to be about 1000–1500 mg.-% of it in normal serum, which would be highly improbable.

A single but broad maximum at 4700 and 4800 Å was found for fructose. The fructose-6-phosphate and Ba 6-phosphogluconic acid gave curves similar to that of fructose, but with less absorption, and the Ba diphosphoketogluconate, and the calcium salt of 2-keto-glu-
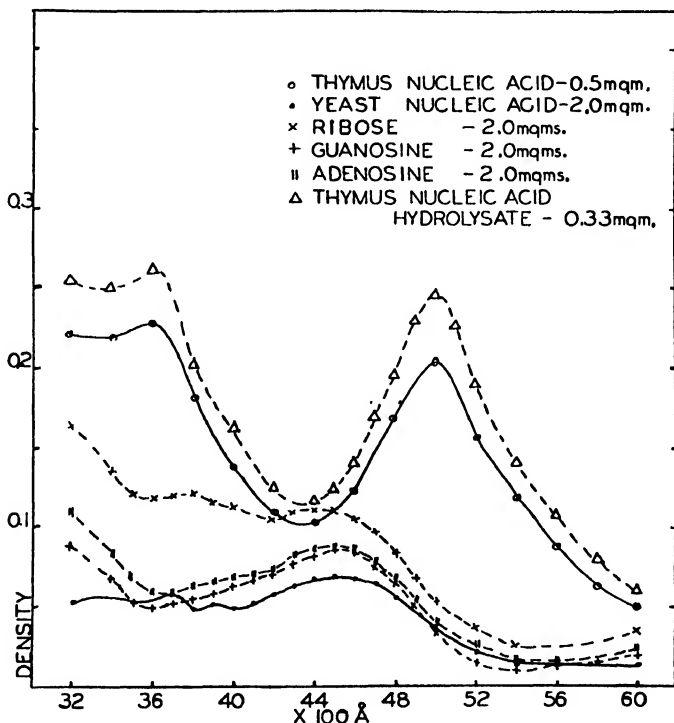
FIG. 4. Spectral absorption curves of perchloric acid condensates of thymus and yeast nucleic acids and their derivatives.

conic acid showed no significant absorption for solutions of comparable concentrations. This absorption of 4700 Å corresponds closely with that of the unknown substance in the serum, and therefore suggests the presence of fructose.

Direct determinations, however, for fructose on the sera of a normal person and a patient with far advanced tuberculosis gave 7.5 and 8.3 mg.-%, respectively, using the method of Corcoran and Page (12).

These values correspond well with the 7 mg.-% blank value for serum found by Alving, Rubin and Miller (13), and are too small to account for the unknown substance in serum. This would seem to rule fructose out.

The possibility still remains that a mixture of ribose, desoxyribose-nucleic acid and derivatives of both may exist in the serum. Such a mixture on reaction with tryptophan and perchloric acid would yield a solution showing a broad absorption maximum around 4700 Å, just as serum does. Moreover, such a mixture could be present in human sera, in view of the findings of Zittle (14) that ribonucleinase exists in the blood and tissues of rats and rabbits. Moreover, his finding (15) that the nucleic acids and their hydrolytic products, the mononucleo-tides, are inhibitory to the nuclease derivatives, suggests the presence in serum of both nucleic acid and its derivatives.

If ribose compounds were present in serum, a green color with a maximum absorption at 6600 Å should be produced with the orcinol reagent (16), but this color did not develop when a test of serum was made. Instead, a reddish brown color with absorption maxima at 4400 and 5600 Å occurred.

Furthermore, if hydrolytic products of either of the nucleic acids were present, they would diffuse away from the serum on dialysis and could be detected in the diffusate by means of ultraviolet absorption. Therefore, two normal sera and two tuberculous sera were dialyzed at 3°C. against equivalent volumes of saline for 48 hrs. Analyses were made on the original and dialyzed sera and also on the diffusates by means of the perchloric acid-tryptophan and carbazole methods. The results showed that very little, or nothing, giving the perchloric acid-tryptophane reaction dialyzed away from either of the two normal sera, whereas 6 and 10 times as much dialyzed away from the pathological sera, respectively. The absorption curves for only one tuberculous and one normal serum are given in Fig. 5, since both experiments gave similar results. If all the substance in the diffusates from the pathological sera which gave the perchloric acid-tryptophan reaction was calculated as nucleic acid, there would be a concentration of at least 4 mgs. ribonucleic acid/ml., or 0.165 mg. desoxyribonucleic acid/ml. Since a concentration of 0.02 mgs./ml. of either of the nucleic acids is sufficient to give a strong spectral absorption at a wave length of 2600 Å, there should be ample material in these diffusates to give a definite absorption at this wave length if this substance is of nucleic acid nature. However, a study of the ultraviolet absorption showed no maximum at 2600 Å, indicating definitely that the diffusible substance is not related to nucleic acids. The maximum at 2850 Å suggested, instead, protein products.

In view of the lack of conclusive evidence as to the exact nature of the substance at present, it seemed advisable, therefore, not to adopt

any particular standard, but, instead, to record the analyses in terms of actual Klett colorimeter readings. The method has been called the TA reaction, an abbreviation for tryptophan-acid reaction. The results are reproducible and appear to have significance for clinical diagnosis, as will be shown below.
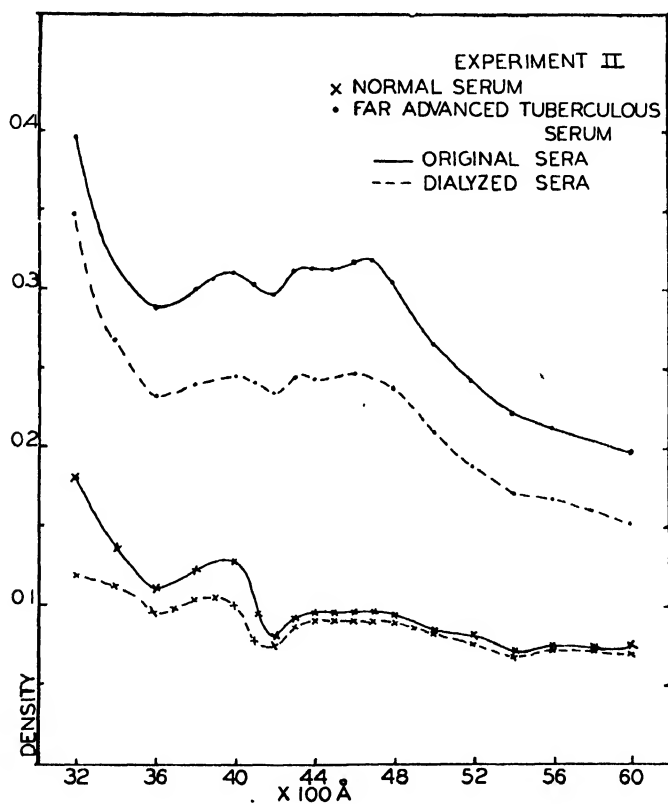
FIG. 5. Spectral absorption curves of perchloric acid condensates of original and dialyzed normal and far advanced tuberculous sera.

In the present report a correlation has been made between the changes in this serum carbohydrate recorded as Klett readings, and the electrophoretic changes in the protein constituents of the serum, of which the α_2-globulin seems to be highly significant.

Analyses of Sera

Sera studied were from patients who were carefully selected and classified as to their diagnosis of tuberculosis, carcinoma, and sarcoidosis, as described in an earlier communication (1). In the case of the tuberculous patients, they were grouped further according to stage of disease. The designations 81, 82 and 83 represent, respectively, minimal, moderately advanced and far advanced active disease. The designations 84 and 85 represent minimal and moderately advanced disease in patients who had recovered to such extent that their lesions were now classified as of questionable clinical significance.

Method

Reagents. Perchloric acid: C.P. 60%.

Saline: 0.9% in distilled water.

Tryptophan: 0.25% of the purest product available. The crystals were put into solution with 0.075 N NaOH (20–26 ml. were used to dissolve 250 mg. tryptophan). This solution was neutralized with N HCl and diluted to 100 ml. with distilled water.

Technique. To 0.25 ml. serum were added 0.75 ml. 0.9% saline, 2.0 ml. 0.25% tryptophan, and 3.0 ml. 60% perchloric acid. Samples were run in duplicate.

Two tubes, as control on the reagents, were run simultaneously; these contained 1.0 ml. 0.9% saline, 2.0 ml. 0.25% tryptophan, and 3.0 ml. 60% perchloric acid.

All tubes were gently shaken, covered with glass stoppers, and immersed simultaneously for exactly 10 mins. in a *vigorously boiling* water bath. They were then removed simultaneously from the bath, cooled in water, and allowed to stand for 40 mins. with occasional shaking to enable the precipitate to coagulate completely. The turbid pinkish brown solutions were then filtered through small (70 mm.) filter papers (Whatman No. 42) until clear. If the solutions are not clear, the results will be too high. To prevent the precipitate from creeping over into the filtrate, the funnel is never filled more than half full. If creeping does occur, the solution must be refiltered.

The solutions were then read in the Klett colorimeter, using the 5000 Å filter, against the reagent controls, which were set at zero.

Since the natural color of various sera differs considerably, controls on the sera were made by testing a solution of 0.25 ml. serum diluted with 5.75 ml. saline. The solutions were not heated, but simply read against a solution of saline.

The results were recorded as Klett readings. The blank readings on the sera were subtracted from the readings on the unknown solutions and the differences recorded as the true readings.

The method is hereafter referred to as TA, meaning the reaction in tryptophan and acid solution.

RESULTS

After the sera were prepared, they were immediately preserved at a temperature just below freezing until analyzed. Analyses made on sera

kept under these conditions showed no change over extended periods of time and results were readily checked within 2–4 Klett divisions. The amount of color produced was directly proportional to the concentration of serum used.

Analyses were made for the TA reaction, as described above under Method. *Polysaccharide* and *glucose* were determined by the carbazole and Somogyi reactions as outlined recently (7). *Total protein* was measured by the Kingsley biuret method, and protein patterns by means of the Tiselius electrophoresis technic as in the former study (1).

Since this study corroborated the former one (1), in that the most significant changes from the normal occurring in tuberculous patients were in the α_2- and γ-globulin fractions and the polysaccharide content, only the data for these components will be recorded in this paper and correlated with the new TA results. Tables III and IV give the mean

TABLE III

Serum Analyses

	No. cases	α_2-Globulin					γ-Globulin				
		Mean g.-%	Range g.-%	σ	$\sqrt{E_1^2+E_2^2}$	Sig.	Mean g.-%	Range g.-%	σ	$\sqrt{E_1^2+E_1^2}$	Sig.
Normal	30	0.77	0.57–0.98	0.11	0.01		1.00	0.70–1.38	0.19	0.02	
Active Tbc.											
Minimal 81	22	0.80	0.63–1.14	0.12	0.02	1.3	1.25	0.73–1.94	0.29	0.04	5.3
Mod. adv. 82	16	0.87	0.73–1.19	0.11	0.02	4.4	1.59	0.98–2.87	0.56	0.09	6.1
Far adv. 83	21	1.19	0.88–1.48	0.17	0.03	14.8	1.81	0.96–3.27	0.61	0.09	8.7
Questionably Active Tbc.											
Minimal 84	14	0.78	0.60 0.94	0.08	0.02	0.5	1.10	0.74–1.40	0.18	0.03	2.6
Mod. adv. 85	13	0.86	0.55–1.53	0.24	0.05	1.9	1.12	0.61–1.56	0.25	0.05	2.3
Carcinoma	15	1.06	0.71–1.39	0.19	0.03	8.1	1.00	0.45–1.63	0.31	0.05	0

"Active lesions" were those which in X-ray films were soft and flocculent, or had shown recent fluctuation, or from which tubercle bacilli had been recently demonstrated in the sputum.

"Lesions of questionable clinical significance" were those with infiltrations that had an X-ray appearance suggesting chronicity or stability, as indicated by fibrosis and scarring in part or parts of the lesion, with softer or less well defined shadows elsewhere suggesting that the disease was in part unhealed.

The numbers 81, 82, 83, 84, and 85 are code numbers at the Henry Phipps Institute, representing, respectively, active tuberculosis of minimal, moderately advanced and far advanced extent, and tuberculosis of questionable clinical significance of minimal and moderately advanced extent.

The carcinoma cases included tumors of the lungs, colon, rectum, caecum, stomach, liver, tongue, breast, and uterus. One brain tumor was included in the series.

TABLE IV

Serum Analyses

	No. cases	(TA) Perchloric acid-tryptophan reaction					Polysaccharide				
		Mean Klett-R	Range Klett-R	σ	$\sqrt{E_1{}^2+E_2{}^2}$	Sig.	Mean mg.-%	Range mg.-%	σ	$\sqrt{E_1{}^2+E_2{}^2}$	Sig.
Normal	30	60	32–79	12.9	1.6		103	78–127	12.6	1.6	
Active Tbc.											
Minimal 81	22	72	53–102	12.8	1.7	*4.9*	108	76–133	14.2	2.0	2.3
Mod. adv. 82	16	91	66–155	24.4	3.9	*7.3*	130	102–191	21.8	3.8	*7.2*
Far adv. 83	21	146	97–216	28.1	4.1	*19.4*	160	120–206	44.5	6.6	*8.6*
Questionably active Tbc.											
Minimal 84	14	61	41–75	10.4	1.8	0.4	113	85–140	14.2	2.5	3.4
Mod. adv. 85	13	77	53–102	15.6	2.6	*5.5*	117	66–159	68.8	11.6	1.3
Carcinoma	15	106	61–139	30.3	5.3	*8.4*	144	91–178	33.5	5.8	*6.9*

values found for α_2- and γ-globulins, and TA and polysaccharide values, and in each case the range, standard deviation (σ), probable error and significance factor (Sig.) are also recorded.

The significance of the difference from the normal mean was calculated from the ratio $\frac{a_1 - a_2}{\sqrt{E_1{}^2 + E_2{}^2}}$, where $a_1 - a_2$ = the difference between the mean of the normal group and that of the group under study, and where $\sqrt{E_1{}^2 + E_2{}^2}$ = the probable error of $a_1 - a_2$. If the ratio is 5, according to Gavett (17), the odds against getting a mean value of a similar group which is outside of the probable error are about 1300 to 1, and the difference is therefore highly significant. Lower ratios indicate that the differences are not sufficiently significant to be of value. The significant ratios are italicized in Tables III and IV.

In corroboration of the earlier results (1) the γ-globulin in this study increased significantly in minimal cases of active tuberculosis, and progressively increased with advance in the disease. A significant increase in the α_2-globulin, as well as polysaccharide, occurred only when the disease became moderately or far advnaced and increased also in the carcinoma cases.

It is of considerable interest that a significant increase in the TA-reacting material appeared in the sera of cases with active minimal tuberculosis (81) and also in the moderately advanced cases of questionable clinical significance (85). The polysaccharide contents, found by means of the carbazole reaction, were not increased in these cases.

The correlation between the content of α_2-globulin and of TA-reacting material is shown in Fig. 6.

The albumin and albumin/globulin ratios decreased significantly in all cases where increases in α_2- and γ-globulin were noted, as described
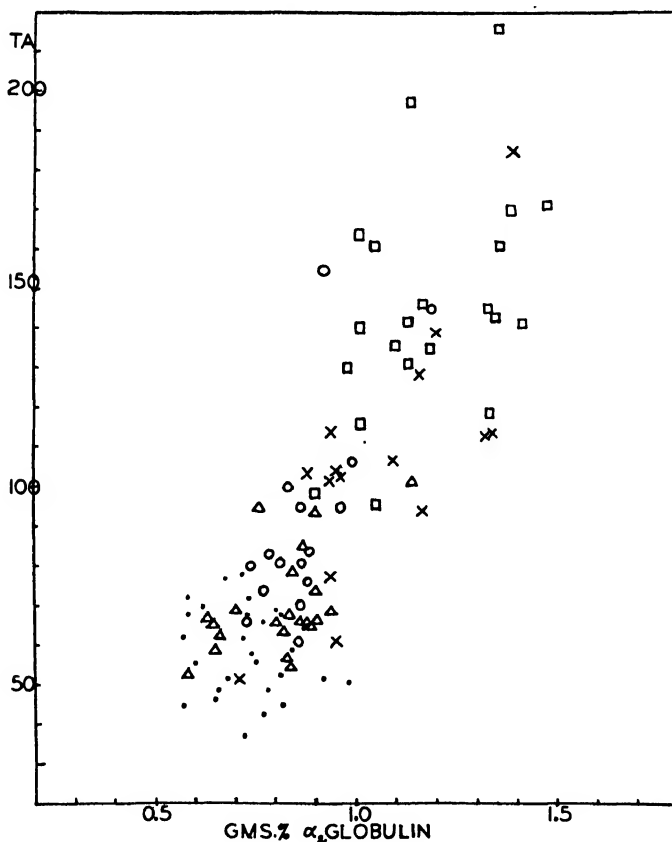
FIG. 6. TA measurements are given in Klett readings. ● Normal sera; △ Minimal active tuberculous sera (81); ○ Moderately advanced active tuberculous sera (82); □ Far advanced active tuberculous sera (83); × Carcinoma sera.

in the former paper, but it was not considered necessary to record all these data.

The sera of 7 cases of sarcoidosis, all of which had been diagnosed by biopsy, showed a significant rise in the β- and especially γ-globulin

constituents, and also in the polysaccharide content and TA substance. The mean TA reading was 95 and the mean polysaccharide content 121 mg.-%.

DISCUSSION

This perchloric acid-tryptophan method promises to be a help in the diagnosis of tuberculosis. Only an extended investigation, including careful correlation with the clinical findings and progress of the disease in individual cases, will be able to establish its value. Similar possibilities should be sought in cases of carcinoma.

The correlation between the TA reaction and the carbazole reaction is of special interest, in that the carbazole reaction measures all carbohydrates, including that in the nucleic acid molecule, whereas the perchloric acid-tryptophan reaction measures chiefly desoxyribose nucleic acid sugar, fructose being a conspicuous exception. The ribose sugars also are detected to a small extent by both reactions. While the sensitivity for detecting minute amounts of carbohydrate of desoxyribonucleic acid is greater, possibly by a factor of nearly 10, in the case of the carbazole reaction, the perchloric acid-tryptophan reaction appears more sensitive in differentiating sugars. This, perhaps, explains why a significant difference was detected between normal sera and sera from cases of minimal tuberculosis by means of the perchloric acid-tryptophan reaction, when no difference was discovered by the carbazole reaction (Table IV).

Some evidence is brought forward in this paper suggesting a relationship between the carbohydrates in serum under question and the carbohydrates of nucleic acid. Both react with perchloric acid and tryptophan on heating. While the absorption maximum for the ribose nucleic acid compounds was at 4600 Å and that of the desoxy compounds was at 5000 Å, a mixture of the two and their derivatives could conceivably have a broad maximum at about 4700 Å–4800 Å, i.e., the same as the maximum for sera. However, the substance which dialyzes away from serum from cases of far advanced tuberculosis, and which gives the perchloric acid-tryptophan reaction, does not give a characteristic absorption in ultraviolet light for nucleic acid or its derivatives.

Fructose also gives this perchloric acid-tryptophan reaction and the spectral absorption of this reaction mixture shows a maximum at the

same wave length as that for serum. However, a direct determination of fructose in the serum failed to show an amount sufficient to account for the amount which would be calculated from the perchloric acid-tryptophan results and, moreover, no significant difference was detectable between normal sera and sera from cases of far advanced tuberculosis. More work must be done to establish the exact nature of this substance.

SUMMARY

A simple method suitable for routine clinical analyses, based on the reaction between a carbohydrate complex in the serum and tryptophan in the presence of perchloric acid, has been utilized to detect the presence of a substance in the sera of tuberculosis and several types of carcinoma. A statistically significant increase over the normal value was found even in cases of active tuberculosis of minimal extent; as the disease progressed to moderately and far advanced stages, this substance progressively increased, in some cases by more than 100%. The mean value for minimal cases which had become of questionable clinical significance was the same as for normals, but that for the moderately advanced cases in this category was increased.

The nature of this substance is unknown. Its measurement, however, should be of value as a supplementary procedure to other laboratory and diagnostic tests in determining the extent and character of certain pathological states.

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Water-Insoluble Nicotinic Acid Esters[1]

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INTRODUCTION

Enrichment of certain staple foods such as corn grits, rice, and macaroni with the water-soluble B vitamins niacin, riboflavin, and thiamine has been complicated by subsequent loss of the vitamins during various water treatments to which these foods are customarily subjected. Two means of minimizing such losses have been suggested. Furter *et al.* (1) apply a solution of the vitamins to rice, and then coat the grains with a water-impervious layer. The second general approach has been to use water-insoluble derivatives of the vitamins concerned, and, in the case of niacin, a variety of such derivatives has been prepared (2, 3).

The present paper describes additional compounds of nicotinic acid which are insoluble in water, but possess the full physiological activity of niacin when tested in the dog. These substances are esters of nicotinic acid with alcohols of the carbohydrate type, particularly glucose.

EXPERIMENTAL

Glyceryl Trinicotinate

Thirty g. of nicotinic acid (0.24 mole) and 74.4 g. of thionyl chloride (0.625 mole) were refluxed for 2 hours and the excess thionyl chloride distilled off. The last traces were removed by heating on the steam bath and evacuating at 15–20 mm. for about 30 minutes. To 11.5 g. (0.065 mole) of the nicotinyl chloride hydrochloride so prepared, were added 30–40 g. of dry pyridine and 1.97 g. (0.0214 mole) of freshly re-

distilled glycerol. The mixture became hot, and was further heated under reflux for 15 minutes to bring all of the solid into solution. The bulk of the pyridine was then removed at reduced pressure, the residue thoroughly extracted with benzene, and the insoluble pyridine hydrochloride filtered off. Concentration of the clear, wine-red benzene filtrate left a red gum, which gradually changed to a pale buff, granular solid when rubbed up thoroughly under absolute ether.

This product was dissolved in benzene, decolorized with activated charcoal, and ether added to the filtrate to incipient turbidity. Crystallization was very slow, although later runs were induced to crystallize more rapidly by seeding. After several days, 5.9 g. (68%) of colorless, thick needles, m.p. 82–84°, were obtained. For analysis a small amount was further recrystallized several times from benzene, m.p. 86–87°C.

$C_{21}H_{17}O_6N_3$. Calculated: C, 61.91; H, 4.21; saponification equivalent, 135.8; found: C, 61.34; H, 4.17; saponification equivalent, 134.3.

The trihydrochloride was precipitated from an ether solution of the free base with HCl gas, and was crystallized several times from ethanol and from ethanol-acetone mixtures, m.p. 192–193°C.

$C_{21}H_{20}O_6N_3Cl_3$. Calculated: Cl, 20.58; found: Cl, 21.47.

D-*Glucose Pentanicotinate*

Fifteen g. (0.084 mole) of nicotinyl chloride hydrochloride were dissolved in 75 cc. of pyridine and a solution of 3 g. (0.0165 mole) of anhydrous D-glucose in 75 cc. of pyridine was added. The mixture was heated at 120°C. until complete solution occurred. The product was worked up as described above, and on removal of the benzene formed a deep orange, viscous syrup. After all attempts to induce crystallization failed, the syrup was exhaustively dried, and analyzed.

$C_{36}H_{27}O_{11}N_5$. Calculated: saponification equivalent, 141.1; found: saponification equivalent 122.1.

In view of the difficulty of purifying the material in its amorphous condition, 0.5 g. was dissolved in a minimum amount of 95% ethanol and a saturated solution of picric acid in 95% ethanol added. The mixture was heated to boiling and the supernatant liquid decanted from a small amount of tarry material. On cooling, yellow crystals which appeared to be the pentapicrate were precipitated, and these were recrystallized 3 times from 50% ethanol, m.p. 116.5–118.5°C.

$C_{66}H_{42}O_{46}N_{20}$. Calculated: C, 42.82; H, 2.29; picric acid, 61.88;[2] found: C, 44.6; H, 2.95; picric acid, 62.1.[2]

Since the material apparently was still not entirely pure, further investigation of the pentanicotinate of D-glucose was discontinued.

α-*Methyl*-D-*Glucopyranoside Tetranicotinate*

To a mixture of 10 g. (0.056 mole) of nicotinyl chloride hydrochloride and 100 cc. of pyridine was added 2.5 g. (0.013 mole) of α-methyl-D-glucopyranoside dissolved in 10 cc. of pyridine. The flask was heated under reflux at 140°C. until the solids had completely dissolved. After working up the reaction mixture as before, the benzene

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[2] Benzene-soluble material obtained after decomposing the picrate with 5 N hydrochloric acid at 100°C.

solution of the crude product was carefully diluted with petroleum ether[3] to incipient turbidity, and allowed to stand overnight in the cold. The colorless, crystalline product was recrystallized to constant m.p. from the same solvent mixture. Five g. (63%), m.p. 137°C., were obtained.

$C_{31}H_{26}O_{10}N_4$. Calculated: C, 60.59; H, 4.26; N, 9.11; found: C, 60.38; H, 4.07; N, 9.06.

D-*Glucose Tetranicotinate*

A suspension of 500 mg. of α-methyl-D-glucopyranoside tetranicotinate in 10 cc. of water containing 2 drops of concentrated HCl was boiled for 5 minutes, and then neutralized with sodium carbonate. The oily product was washed twice with water by decantation, dissolved in benzene, and brought to crystallization by the careful addition of petroleum ether. Recrystallization from ether-petroleum ether gave 370 mg. (76%), m.p. 141–142°C.

$C_{30}H_{24}O_{10}N_4$. Calculated: C, 60.00; H, 4.03; found: C, 60.03; H, 4.03.

D-*Galactose Pentanicotinate*

Three g. (0.017 mole) of anhydrous D-galactose was esterified with 17 g. (0.095 mole) of nicotinyl chloride hydrochloride and the mixture worked up as described above. All attempts to crystallize the product failed. It was, therefore, converted to the picrate, which was purified as in the case of the glucose pentanicotinate. The product (apparently the tetra picrate) formed yellow crystals, m.p. 123°C.

$C_{60}H_{39}O_{39}N_{17}$. Calculated: C, 44.43; H, 2.42; picric acid, 56.50; found: C, 44.14; H, 2.94; picric acid, 57.0.

TABLE I

Microbiological Assay of Various Nicotinic Acid Esters

Substance	Pretreatment	Nicotinic acid	
		Found	Calculated
		mg./g.	*mg./g.*
Glyceryl trinicotinate	None	702	907
	Acid-autoclaved	943	907
D-Glucose pentanicotinate[a]	None	523	872
	Acid-autoclaved	753	872
D-Galactose pentanicotinate[a]	None	497	872
	Acid-autoclaved	785	872
α-Methyl-D-glucopyranoside tetra-nicotinate	None	263	802
	Acid-autoclaved	768	802
D-Glucose tetranicotinate	None		821
	Acid-autoclaved	750	821

[a] Amorphous preparation.

[3] The petroleum ether used was commercially purified material, b.p. 60–80°, sold under the name "Skelly-Solve B."

Biological Activity

The potency of the above products as sources of nicotinic acid was tested microbiologically, and, in some cases, with the dog. Results of the microbiological tests, which were carried out with *Lactobacillus arabinosus* by the standard nicotinic acid assay technique (4), are given in Table I. For assay, dilute alcoholic solutions of the various compounds were further diluted to the desired concentration with water. Acid autoclaving was carried out as specified in the assay procedure (4).

The dog assays were carried out according to the method of Elvehjem *et al.* (5), with the results shown in Table II. With one exception each compound was tested in a niacin-deficient dog whose weight response to a small dose of nicotinic acid had been just previously determined. The comparative responses are shown in Table II. In the case of α-methyl-D-glucopyranoside tetranicotinate, however, two groups of 4 dogs each, maintained as nearly as possible in comparable states of niacin deficiency, were separately given the pure vitamin (dogs 448, 449, 450, and 451) and the test substance (dogs 120, 441, 442, and 443).

TABLE II
Biological Activity of Nicotinic Acid Esters for Dogs

Dog no.	Compound fed	Dose	Molar equivalent of nicotinic acid	Maximum weight gain	Original weight
		mg.	*mg.*	*kg.*	*kg.*
119	Nicotinic acid	25	25	0.75	2.75
119	Glyceryl trinicotinate	35	31.2	1.5	3.2
109	Nicotinic acid	25	25	1.5	7.25
109	Glyceryl trinicotinate	35	31.2	1.6	8.5
448	Nicotinic acid	20	20	0.9	3.4
449	Nicotinic acid	20	20	1.0	5.3
450	Nicotinic acid	20	20	0.9	4.8
451	Nicotinic acid	20	20	0.6	4.1
120	α-Methyl-D-glucopyranoside tetranicotinate	25	20	0.7	9.9
441	α-Methyl-D-glucopyranoside tetranicotinate	25	20	1.1	5.8
442	α-Methyl-D-glucopyranoside tetranicotinate	25	20	0.5	4.3
432	α-Methyl-D-glucopyranoside tetranicotinate	25	20	1.0	11.6
119	Nicotinic acid	25	25	0.95	4.9
119	D-Glucose pentanicotinate[a]	35	30.5	0.9	5.6
419	Nicotinic acid	25	25	1.0	3.7
419	D-Glucose pentanicotinate[a]	35	30.5	0.9	5.0
420	Nicotinic acid	25	25	1.0	3.2
420	D-Galactose pentanicotinate[a]	35	30.5	1.4	3.8

[a] Amorphous preparation.

ENRICHMENT OF RICE

The nicotinic acid esters prepared in this study were found to be soluble in such organic solvents as benzene, chloroform, alcohol, and acetone, and also in cold, dilute, aqueous mineral acid solutions. The alcohol and acetone solutions were precipitated by dilution with water, and the acid solutions by neutralizing.

To test in a preliminary way the suitability of such substances for enrichment purposes, one compound, α-methyl-D-glucopyranoside tetranicotinate, was applied to polished rice grains and tested for its resistance to leaching with water. Two methods of application were used. For the first, a 4% solution of the tetranicotinate in 95% alcohol was sprayed with a hand atomizer on a quanitty of rice in a wide shallow dish. The rice was stirred continuously and the solution was applied slowly to allow the alcohol to evaporate.

In the second procedure the rice was covered with a 0.5% solution of the tetranicotinate in dilute HCl, just sufficient acid being used to dissolve the ester, and soaked for 3 hours at room temperature. The excess solution was then decanted, and the rice was covered for 1 hr. with 5% sodium bicarbonate solution. The rice was finally drained, and dried in the oven. The appearance of the grain was not appreciably altered by either treatment.

Samples of the enriched rice were then acid-autoclaved and the nicotinic acid potency determined microbiologically. Other samples were subjected to various water treatments, and then assayed in the same manner. The results are collected in Table III.

TABLE III

Retention of Niacin in Enriched Rice after Various Water Treatments

Sample	Treatment	Niacin content[a]	Niacin retained
		mg./g.	*Per cent*
Enriching compound [b] applied on the surface by spraying on an alcoholic solution		
1	None	6.50	100
2	Boiled 30 mins. in 11 parts of water, and drained.	5.90	91
3	Sample 2 washed in cold running water until granules were separate. ·	2.86	44
Enriching compound applied by soaking in acid solution and then neutralizing		
4	None	1.67	100
5	Boiled in water, drained, and washed in cold water.	1.60	96

[a] Dry basis.

[b] α-Methyl-D-glucopyranoside tetranicotinate.

DISCUSSION

The data in Table I indicate that the nicotinic acid esters of glycerol, D-glucose, α-methyl-D-glucopyranoside, and D-galactose are not completely available to *L. arabinosus* as sources of nicotinic acid. However, acid-autoclaving, as would be expected, liberates the vitamin, and substantially theoretical amounts (within the limits of error of the assay method) were then found in all preparations except those of glucose and galactose pentanicotinates which we had been unable to purify completely.

The dog, on the other hand, appears able to utilize the esters as well as an equivalent quantity of free niacin. The difference is undoubtedly due to the hydrolysis of the esters in the animal organism, and the failure of this hydrolysis in the bacteria. This behavior is to be expected, since various other esters of nicotinic acid, riboflavin, and pantothenic acid are inactive toward lactic acid bacteria, but are well utilized by animals (6, 7, 8).

The analysis of the cooked rice samples showed considerable loss when the enriching compound, α-methyl-D-glucopyranoside tetranicotinate, was applied to the surface of the granule, probably because of mechanical removal of a part of the entire surface material caused by the vigorous washing of the cooked rice. The fact that very little loss occurred in the cooking water itself indicates that the ester did not dissolve appreciably, even in the large volume of water used.

When the tetranicotinate was applied by soaking the grain in a dilute acid solution, which was subsequently neutralized, the compound undoubtedly penetrated the interior of the granules, and was precipitated *in situ*. The cooking losses, consequently, were negligible.

It would appear, therefore, that nicotinic acid derivatives of the type described would be satisfactory for food enrichment purposes from the standpoint of stability toward water leaching. In view of their composition and presumable hydrolysis to nicotinic acid plus glycerol or a sugar *in vivo*, it is difficult to suppose that they would be in any way harmful to the animal or human organism. They appear to be active as sources of niacin, and on the weight basis have 80–90% of the potency of nicotinic acid itself.

SUMMARY

A series of esters of nicotinic acid with glycerol and two simple sugars has been prepared. Substances of this type are available to the dog as sources of nicotinic acid, and are insoluble in water. They may be incorporated into foods such as rice, and in preliminary trials do not appear to be extensively removed by subsequent cooking in water.

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Synthesen von Oxy- und Ketofettsäuren

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EINLEITUNG

Mittlere und höhere Oxy- und Ketofettsäuren sind, abgesehen von den aus natürlichen 9,10-ungesättigten Fettsäuren durch Oxydation an den Doppelbindungen erhältlichen Oxyfettsäuren, bisher nicht systematisch synthetisiert worden; da sie weder technisch noch biologisch Interesse boten. Sie treten erst neuerdings in den Vordergrund, seit die Untersuchung der Biochemie der Fettsäuren ihre Synthese verlangt.

So sind von Thaler und Geist (1), Lang und Adickes (2), Breusch und Tulus (3), Adickes und Andresen (4) und anderen α-Ketofettsäuren und β-Oxyfettsäuren, von Breusch, Keskin, Tulus und Ulusoy (5,6,7,8,9) α,γ-Diketofettsäuren, γ,ζ-Diketofettsäuren, β-Ketofettsäuren und α,β-Dioxyfettsäuren synthetisiert worden. Andere Einzelsynthesen stammen von Levene und Haller (10), Ponzio (11), Robinet (12), Simowski (13) und anderen. Bergström, Theorell, Davide (14) isolierten aus *Pseudomonas pyocyanea* β-Oxydecansäure.

Biochemisch interessant sind besonders solche Säuren, die Hydroxyl-oder Ketogruppen in α-, β- und γ-Position tragen, da Breusch und Tulus (15) zeigen konnten, dass, wenigstens bei kurzfristigen Bebrütungsversuchen mit frischem Gewebebrei, nur α- und β-Ketofettsäuren, nicht aber γ- und δ-Ketofettsäuren umgesetzt werden.

EXPERIMENTELLES

In der vorliegenden Arbeit wird die Darstellung einiger α-Oxy-γ-Ketofettsäuren sowie einiger α-Oxyfettsäuren durch partielle Reduktion der nach Breusch und Keşkin (5) dargestellten α,γ-Diketofettsäuren mit nicht amalgamiertem Zink und HCl beschrieben.

$$\begin{array}{cccccc} & & H & H & H & \\ R & - & C - C - C - C & - & COOH \\ & & | & \| & | & | \\ & & H & O & H & OH \end{array}$$

Formel II
α-Oxy-γ-ketofettsäuren

$$\begin{array}{cccc} & H & H & \\ R-C-C-C-C & - & COOH \\ | & \| & | & \| \\ H & O & H & O \end{array}$$

Formel I
α,γ-Diketofettsäuren

$$\begin{array}{ccccc} & H & H & H & H \\ R-C-C-C-C & - & COOH \\ | & | & | & | \\ H & H & H & OH \end{array}$$

Formel III
α-Oxyfettsäuren
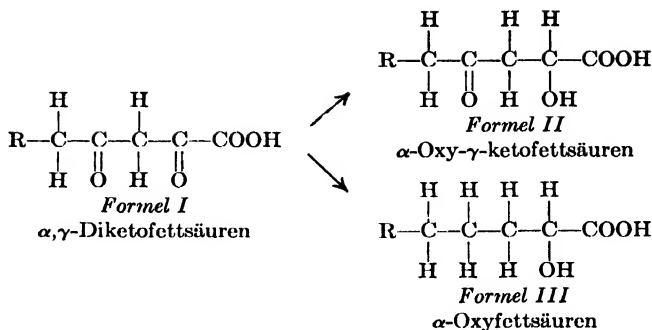
Auch bei Verwendung von amalgamiertem Zink nach Clemmensen tritt keine Totalreduktion der Ketogruppe ein, da nach Komppa (16), Steinkopf und Wolfram (16a) α-, β- und γ-Ketogruppen infolge sterischer Hemmungen nur schwer zu CH₂-Gruppen reduzierbar sind. Bei dieser Reduktion entstehen dementsprechend 50–80% ölige Produkte, die wir nach vergeblichen Trennungsversuchen nicht mehr weiter bearbeitet haben; die wahrscheinlich γ-Lactongemische von α-Oxy-γ-Oxy, von α-Keto-γ-oxy-, und von γ-Oxyfettsäuren sind.

Weiter wurden die α,β-ungesättigten γ-Ketofettsäuren nach der Methode von Koenigs und Wagstaffe (17) durch Kondensation von Chloral mit Methylketonen zu Chloralketonen und nachfolgende Verseifung dargestellt. In diesen Verbindungen lässt sich die α,β-Doppelbindung überraschenderweise mit Zn und HCl aushydrieren. Wir erhielten so die entsprechenden γ-Ketofettsäuren. Durch Oxydation der ungesättigten Ketofettsäuren mit kalter Permanganatlösung nach Fittig (18) und Lapworth, Mottram (19) liessen sich die α,β-Dioxy-γ-ketofettsäuren darstellen.

Formel VI
γ-Ketofettsäuren
↑ Reduktion

Methylketon + Chloral → 1-Trichlor-2-Oxy-4-ketoparaffin Chloralketon (Formel IV) → α,β-ungesättigte-γ-Ketofettsäure (Formel V)

Formel V

↓ Oxydation

Formel VII
α,β-Dioxy-γ-ketofettsäure
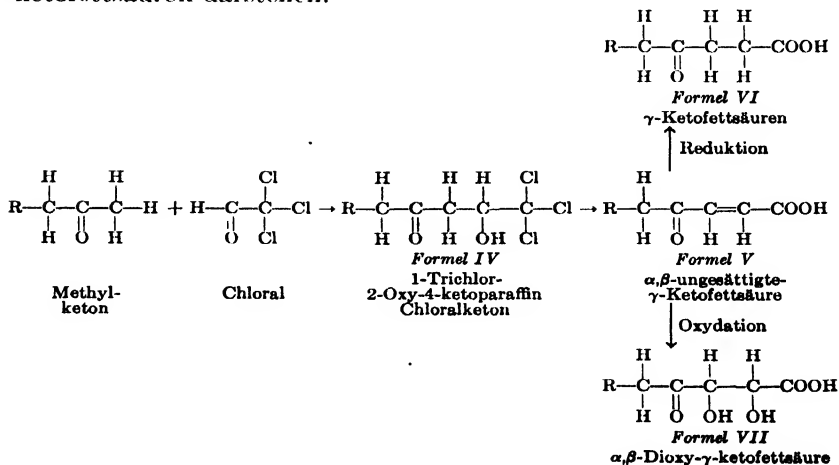
Von den meisten Säuren (von vielen hatten wir nur wenige Milligramm) wurden kristallisierte Derivate hergestellt: Semicarbazone, Oxime oder Ureide mit bis(p-dimethylaminophenyl)carbodiimid nach Breusch und Ulusoy (20). Eine Spaltung der racemischen Oxyfettsäuren in optisch aktive Komponenten wurde nicht versucht, nachdem schon früher unternommene Versuche, β-Oxylaurinsäure über Alkaloidsalze zu spalten, zu keinem brauchbaren Resultat geführt hatten.

In der grossen Tabelle sind die bis jetzt bekannten α,β,γ-Oxy und Ketofettsäuren verzeichnet. Die erste Zahl gibt den Schmelzpunkt, die eingeklammerte Zahl das Literaturzitat. In dieser Arbeit neu beschriebene Substanzen sind mit * bezeichnet.

Nach einem Referat (32) sind von Nolte in einer noch unveröffentlichten Arbeit viele γ-Ketofettsäuren synthetisiert worden; die dort nicht angegebenen Schmelzpunkte sind nach brieflicher Mitteilung von Dr. Nolte, Höchst a.M., eingesetzt (N). Nach brieflicher Mitteilung von Dr. Stenhagen, Uppsala, sind von M. Skogh eine Reihe von höheren β-Oxyfettsäuren synthetisiert worden, deren Schmelzpunkte als (S.S.) eingesetzt sind. Die Arbeit erscheint in *Scand. Chim. Acta.*

Reduktion der α,β-Diketofettsäuren (Formel I)

Die α,γ-Diketofettsäuren wurden nach Breusch und Keskin (5) dargestellt. Zur Reduktion wurden wegen ihrer Zersetzlichkeit nicht die freien Säuren, sondern ihre Äthylester verwendet.

5 g. Ester würden mit 20 ml. Äthanol, 10 g. granuliertem, nicht amalgamiertem Zink und 10 ml. konz. HCl bei Zimmertemperatur mit einem Motorrührer solange gerührt, bis die für die α-Ketogruppe charakteristische Rotfärbung mit FeCl$_4$ gerade verschwand. Bei Bedarf wurden weitere kleine Mengen Äthanol und HCl zugesetzt. Die Reaktion dauert 2–5 Stunden. Dann wurde mit 50 ml. H$_2$O versetzt und ausgeäthert. Der abgetrennte, eingedampfte Ätherextrakt wurde mit dem doppelten der nötigen Menge 10%iger NaOH-Lösung in H$_2$O bei Zimmertemperatur 30 Minuten verrührt. Dann wurde mit Äther die geringe Menge des nicht verseiften Esters entfernt, die abgetrennte alkalischwässrige Lösung angesäuert und neuerdings ausgeäthert. Der abgetrennte, mit wasserfreiem Na$_2$SO$_4$ getrocknete Äther wurde verdampft. Der Rückstand besteht nach dem Erkalten aus öligen Kristallen. Sie werden in möglichst wenig Benzol heiss gelöst und nach dem Erkalten mit Petroläther vorsichtig tropfenweise bis zur Trübung versetzt. Beim Abkühlen im Eisschrank auf −17° kristallisieren die α-Oxy-γ-Ketofettsäuren (Formel II) in 10 bis maximal 40%iger Ausbeute aus.

Setzt man die Reduktion statt mit gewöhnlichem Zink mit amalgamiertem Zink und HCl mehrere Tage fort, so erhält man statt der α-Oxy-γ-ketofettsäuren in geringer Ausbeute kristallisierte α-Oxyfettsäuren (Formel III); neben grossen Mengen nicht kristallisierender Öle, die wahrscheinlich γ-Lactongemische darstellen.

Struktur (Gesamtkettenlänge)	C$_5$	C$_6$	C$_7$	C$_8$	C$_9$	C$_{10}$	C$_{11}$	C$_{12}$	C$_{13}$	C$_{14}$
HOOC–C–C–C–C–C–C–R (H H O H H O H)	—	—	—	76° (31)	85° (9)	84° (9)	85° (9)	93° (9)	—	—
Cl–C–C–C–C–C–R (Cl OH H O H)	76° (17)	58° *	51° *	45,5° *	54,5° *	46° *	40° *	—	—	—
HOOC–C–C–C–R (HO OH O H)	—	—	—	—	122° *	123° *	—	—	—	—
COOH–C=C–C–C–R (O H)	126° (17)	109° *	112° *	106° *	118° *	111° *	—	—	—	—
HOOC–C–C–C–R (O H)	104° (30)	—	—	73° *	81° *	83° *	—	89° *	—	—
HOOC–C–C–C–R (O H O H)	98° (29)	83° (5)	56° (5)	45° (5)	52° (5)	51° (5)	50° (5)	53° (5)	62° (5)	—
HOOC–C–C–C–R (H H O H)	31°	37° (28)	47° (28)	53° *(28)(N)	69° *(N)	64° *	—	—	87° (N)	87° (N)
HOOC–C–C–C–R (H O H H)	—	—	—	74° (27)	—	85° (6)	75° (6)	—	65° (6)	—
HOOC–C–C–C–R (O H H H)	7° (4)	8° (4)	30° (4)	33° (4)	44° (4)	47° (4)	55° (4)	57° (4)	62,5° (4)	64° (4)
COOH–C–C–C–R (HO H HO H)	—	—	—	—	121° (8)	123° (8)	125° (8)	126° (8)	126° (8)	127° (8)
HOOC–C–C–C–R (H HO H H)	44° (4)	13° (4)	41° (4)	38,5° (4)	60° (4)	56° (4,1)	73° (4,3)	79° (4,3)(S.S.)	81° (3)	73°, 78° (1,3)(S.S.)
HOOC–C–C–C–R (HO H H H)	34° (21)	60° (22)	65° (23)	69° (23)	70° (24)	70,5° *(25)	69° (25)	74° (24)	78° (26)	82° (26)

Struktur	C₁₅	C₁₆	C₁₇	C₁₈	$R=-C=C<\!\!\begin{smallmatrix}CH_3\\H\end{smallmatrix}$	$R=-C<\!\!\begin{smallmatrix}CH_3\\H\end{smallmatrix}$	$R=-C-C-$
HOOC–C–C–C–C–C–R	—	—	—	—	—	—	—
Cl₂C–C–C–C–C–R	—	—	—	90° *	87° *	78° *	
HOOC–C–C–C–C–R	—	—	—	—	— | *	—	
HOOC–C=C–C–C–R	—	—	—	—	—	92° *	103° *
HOOC–C–C–C–C–R	92° *	—	—	—	—	—	—
HOOC–C–C–C–C–R	70° (5)	—	—	—	167° (7)	49° (7)	—
HOOC–C–C–C–C–R	—	91° (N)	—	96° (N)	—	47° (28a)	51° *
HOOC–C–C–C–C–R	—	—	—	—	—	—	—
HOOC–C–C–C–C–R	68.5° (4)	69° (4)	—	—	—	—	—
HOOC–C–C–C–C–R	—	127° (13)	—	126° (11)	—	106° (28a)	—
HOOC–C–C–C–C–R	87°* (S.S.)	83.5°,84.5° (12)(S.S.)	91° (S.S.)	89°,90° (11)(S.S.) (10)	—	37° (33)	—
HOOC–C–C–C–C–R	84° (26)	87° (26)	89° (26)		—	91° (S.S.)	—

α-Oxy-γ-Ketononansäure (Formel II; R = —CH₂—CH₂—CH₂—CH₃),

α-Oxy-γ-Ketononansäure (Formel II; R = —CH$_2$—CH$_2$—CH$_2$—CH$_3$),

Farblose Krist. Smp. 71–73°. Ausbeute 15%. Gibt warm Fehlingreaktion. Lösl. in Äthanol, Äther, Benzol, unlösl. in Petroläther.

C,H-Bestimmung. C₉H₁₆O₄: Ber.: C = 57,44%; H = 8,61%
Gef.: C = 57,38%; H = 8,63%.

α-Oxy-γ-Ketodecansäure

(Formel II; R = —CH₂—CH₂—CH₂—CH₂—CH₃)

Farblose Kristalle; Smp. 79–81°. Ausbeute 20%. Gibt warm Fehlingreaktion. Löslich in Äthanol, Äther, Benzol, 0,5% lösl. in H₂O; unlöslich in Petroläther.

C,H-Bestimmung. C₁₀H₁₈O₄: Ber.: C = 59,40%; H = 8,10%
Gef.: C = 59,36%; H = 8,17%.

NaOH-Titration. Molekulargewicht ber. 202; gef. 201.

Durch Oxydation der *α*-Oxy-*γ*-ketodecansäure mit KMnO₄ in saurer Lösung konnte Heptylsäure als bei 133° schmelzendes Ureid mit Bis (*p*-dimethylaminophenyl) carbodiimid nach Breusch und Ulusoy (20) isoliert werden.

Versuche, ein kristallisiertes Ureid der Säure selbst herzustellen, führte nur zu schwarzen Produkten, aus denen kleine Mengen von über 200° sich zersetzenden Kristallen isoliert werden konnten. Mit Phenyl-, Nitrophenyl- und Dinitrophenylhydrazin wurden nur ölige Produkte erhalten.

Semicarbazon. Aus der gesättigten wässrigen Lösung mit Semicarbazidchlorhydrat nach einer Stunde als weisse Kristalle; aus Benzol und Petroläther umkristallisierbar. Smp. 115°, unlösl. in Petroläther und CCl₄.

C,H,N-Bestimmung. C₁₁H₂₁O₄N₃; Ber.: C = 50,96%; H = 8,10%; N = 16,21%
Gef.: C = 50,65%; H = 7,85%; N = 16,10%.

Lässt man die Säure mehrere Tage mit überschüssigem Semicarbazidchlorhydrat stehen, so bilden sich, wahrscheinlich unter Wasserabspaltung und Cyklisierung, Kristalle vom Smp. 220°.

N-Bestimmung (Dumas): C₁₁H₁₉O₃N₃: Ber. N = 17,4%; Gef. N = 17,1%.

Oxim. Kristalle aus gesättigter wässriger Lösung nach einer Stunde. Aus H₂O umkristallisierbar. Smp. 96°. Lösl. in Äthanol, Aceton; unlösl. in CCl₄.

C,H-Bestimmung. C₁₀H₁₉O₄N: Ber. C = 55,30%; H = 8,75%
Gef. C = 55,42%; H = 8,59%.

α-Oxy-γ-Ketoundecansäure

(Formel II; R = —CH₂—(CH₂)₄—CH₃)

Farblose Kristalle. Smp. 83°. Ausbeute 40%. Reduziert in der Wärme Fehling. Löslich in Äthanol, Aceton; Löslichkeit in Äther 18%, in H₂O 0,25%, in Petroläther 0,01%.

C,H-Bestimmung. C₁₁H₂₀O₄: Ber.: C = 61,11%; H = 9,25%
Gef.: C = 60,70%; H = 9,10%.

α-Oxy-γ-Ketotridecansäure
(Formel II; R = —CH₂—(CH₂)₆—CH₃)

Farblose Kristalle; Smp. 89°. Ausbeute 40%; reduziert warm Fehlinglösung. Löslichkeit: gut in Äthanol, Äther; fast unlösl. in H_2O; unlösl. in Petroläther.

C,H-Bestimmung. $C_{13}H_{24}O_4$: Ber.: C = 63,93%; H = 9,83%
Gef.: C = 63,30%; H = 9,50%.

α-Oxy-γ-Ketopentadecansäure
(Formel II; R = —CH₂—(CH₂)₈—CH₃)

Farblose Kristalle; Smp. 92°. Ausbeute 30%; reduziert warm Fehlinglösung. Löslichkeit wie oben.

C,H-Bestimmung. $C_{15}H_{28}O_4$: Ber.: C = 66,17%; H = 10,29%
Gef.: C = 65,78%; H = 11,13%.

α-Oxydecansäure (Formel III; R = —CH₂—(CH₂)₇ CH₃)

wurde durch 15 stündige Reduktion von 2,5 g. freier α,γ-Diketodecansäure mit überschüssigem amalgamiertem Zink in 15%iger HCl bei Zimmertemperatur unter mechanischem Rühren in 100 mg. Ausbeute erhalten. Nach 3-maligem Umkristallisieren aus Petroläther Smp. 69–70°; nach Bagard (25) Smp. 70,5°. Im Gegensatz zu den α-Oxy-γ-ketofettsäuren gibt diese α-Oxyfettsäure keine Fehlingreaktion. Die C,H-Bestimmung ergab richtige Werte. Das farblose Ureid mit Bis (p-dimethylaminophenyl) carbodiimid schmolz nach Umkrist. aus Äthanol bei 134°.

Darstellung von Chloralketonen (Formel IV)

erfolgt nach Koenigs und Wagstaffe (17) durch 3-15 stündiges Erhitzen von wasserfreiem Chloral mit der äquivalenten Menge Methylketon, gelöst in 2 Äquivalenten Eisessig am Rückflusskühler. Die Reaktionslösung wird nach dem Erkalten in überschüssige 4%ige eisgekühlte NaOH-Lösung gegossen und ausgeäthert. Beim Eindampfen des Ätherrückstandes kristallisieren nach 24 stündigem Stehen manche Chloralketone direkt und können dann durch Umkristallisieren aus Benzol und Petroläther rein gewonnen werden. Einige nicht kristallisierende wurden vorher durch Vakuumdestillation vorgereinigt und dann umkristallisiert.

1-Trichlor-2-Oxy-4-Ketohexan
(Chloraläthylketon; Formel IV; R = —CH₃)

Zur Kondensation 15 Stunden am Rückfluss gekocht. Farblose Kristalle; Smp. 56–58°. Siedep.₈ mm Hg 139–140°. Leichtlöslich in $CHCl_3$, Benzol, Äthanol; wenig löslich in Petroläther; unlöslich in H_2O.

C,H-Bestimmung. $C_6H_9O_2Cl_3$; Ber.: 32,80% C; 4,10% H.
Gef.: 33,45% C; 4,02% H.

1-Trichlor-2-Oxy-4-Ketoheptan

(Chloralpropylketon; Formel IV; R = —CH$_2$—CH$_3$)

15 Stunden kondensiert. Farblose Kristalle; Smp. 49–51°. Siedep.$_{12\ mm\ Hg}$ 147–150°. Ausbeute 14%. Löslichkeit wie oben.

C,H-Bestimmung. C$_7$H$_{11}$O$_2$Cl$_3$; Ber.: C = 35,97%; H = 4,71%
Gef.: C = 36,13%; H = 4,95%.

1-Trichlor-2-Oxy-4-Ketooctan

(Chloralbutylketon; Formel IV; R = —CH$_2$—CH$_2$—CH$_3$)

15 Stunden kondensiert. Farblose Kristalle; Smp. 44–45,5°. Siedepunkt$_7$ $_{mm\ Hg}$ 148–151°. Ausbeute 15%. Löslichkeit wie oben.

C,H-Bestimmung. C$_8$H$_{13}$O$_2$Cl$_3$; Ber.: C = 38,78%; H = 5,25%
Gef.: C = 39,23%; H = 5,78%.

1-Trichlor-2-Oxy-4-Keto-6-Methylheptan

$$\text{(Chloralisobutylketon; Formel IV; R} = -\text{CH} \begin{cases} \nearrow \text{CH}_3 \\ \searrow \text{CH}_3 \end{cases}.$$

15 Stunden kondensiert. Farblose Kristalle Smp. 86–87°. Ausbeute 32%. Kristallisiert sehr gut. Löslichkeit wie oben.

C,H-Bestimmung. C$_8$H$_{13}$O$_2$Cl$_3$; Ber.: C = 38,78%; H = 5,25%; Cl = 43,30%
Gef.: C = 38,89%; H = 5,45%; Cl = 43,39%.

1-Trichlor-2-Oxy-4-Keto-6-Methylhepten (5);

Chloralisobutylidenketon;

$$\underset{\underset{\text{Cl}}{|}}{\overset{\overset{\text{Cl}}{|}}{\text{Cl}-\text{C}}} - \underset{\underset{\text{OH}}{|}}{\overset{\overset{\text{H}}{|}}{\text{C}}} - \underset{\underset{\text{H}}{|}}{\overset{\overset{\text{H}}{|}}{\text{C}}} - \underset{\underset{\text{O}}{\|}}{\overset{\overset{\text{H}}{|}}{\text{C}}} - \underset{}{\overset{\overset{\text{H}}{|}}{\text{C}}} = \text{C} \begin{cases} \nearrow \text{CH}_3 \\ \searrow \text{CH}_3 \end{cases}.$$
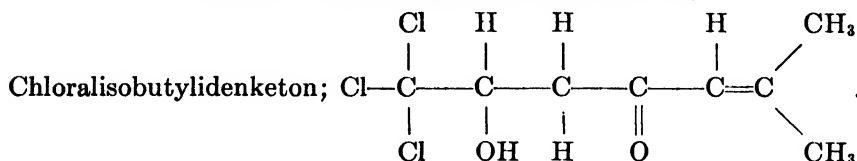
Zur Kondensation 3 Stunden am Rückfluss gekocht. Dunkelfärbung. Nach Umkristallisation farblose Kristalle; Smp. 89–90°. Ausbeute 10%.

C,H-Bestimmung. C$_8$H$_{11}$O$_2$Cl$_3$; Ber.: C = 39,10%; H = 4,48%
Gef.: C = 39,25%; H = 4,35%.

1-Trichlor-2-Oxy-4-Ketononan

(Chloralamylketon; Formel IV; R = —(CH$_2$)$_3$—CH$_3$)

15 Stunden kondensiert. Farblose Kristalle; Smp. 53–54,5°. Ausbeute 20%.

C,H-Bestimmung. C$_9$H$_{15}$O$_2$Cl$_3$; Ber.: C = 41,30%; H = 5,73%
Gef.: C = 40,95%; H = 5,55%.

1-Trichlor-2-Oxy-4-Keto-7-Methyloctan;

Chloralisoamylketon; Formel IV; R = —CH_2—CH $\begin{array}{c} CH_3 \\ \diagup \\ \diagdown \\ CH_3 \end{array}$.
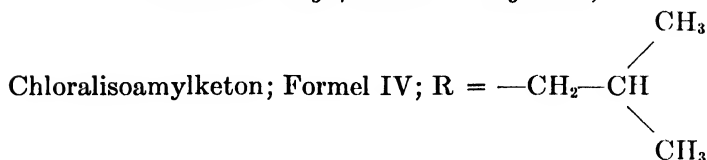
15 Stunden kondensiert. Farblose Kristalle; Smp. 76–78°. Ausbeute 15%. Löslichkeiten wie oben.

C,H-Bestimmung. $C_9H_{15}O_2Cl_3$; Ber.: C = 41,30%; H = 5,73%
Gef.: C = 41,14%; H = 5,72%.

1-Trichlor-2-Oxy-4-Ketodecan

(Chloralhexylketon; Formel IV; R = —$(CH_2)_4$—CH_3)

15 Stunden kondensiert. Farblose Kristalle; Smp. 45–46°. Ausbeute 20%. Löslichkeiten wie oben.

C,H-Bestimmung. $C_{10}H_{17}O_2Cl_3$; Ber.: C = 43,79%; H = 6,20%
Gef.: C = 43,69%; H = 6,18%.

1-Trichlor-2-Oxy-4-Ketoundecan

(Chloralheptylketon; Formel IV; R = —$(CH_2)_5$—CH_3)

15 Stunden kondensiert. Farblose Kristalle; Smp. 38–40°. Ausbeute 18%. Löslichkeiten wie oben; in Petroläther ziemlich löslich.

C,H-Bestimmung. $C_{11}H_{19}O_2Cl_3$; Ber.: C = 45,59%; H = 6,56%
Gef.: C = 45,24%; H = 6,53%.

Die *Verseifung der Chloralketone* zu $\Delta^{\alpha,\beta}$-γ-Ketofettsäuren wurde nach Koenigs und Wagstaffe (17) ausgeführt, deren Vorschrift wir nach unseren Bedürfnissen modifizierten. Die Verseifung wurde entweder durch 1–2 Minuten langes direktes Erhitzen von 2 g. Chloralketon mit 20 ml. Äthanol und 20 ml. 10%iger wässriger KOH, oder durch 10–15 Minuten langes Erhitzen von 2 g. Chloralketon mit 120 ml. 4%iger K_2CO_3-Lösung bewerkstelligt. Nach dem Erkalten wurde der ungelöste, nicht verseifte Teil des Chloralketons der Lösung mit Äther entzogen, die abgetrennte, eventuell filtrierte klare alkalische Lösung mit konz. HCl angesäuert und neuerdings mehrfach ausgeäthert. Der nach dem Abdestillieren des Äthers verbleibende Rückstand wird aus Benzol oder Chloroform durch Zugabe von niedrigsiedendem Petroläther mehrfach umkristallisiert. Ausbeute 20–50%.

Bei der Verseifung spaltet sich aus der α,β-Stellung zur —CCl_3 Gruppe immer 1 Mol H_2O ab. Wir konnten in keinem Fall die zu erwartende α-Oxy-γ-ketofettsäure isolieren, sondern fanden neben öligen, nicht weiter untersuchten Produkten immer nur die α,β-ungesättigten γ-Ketofettsäuren.

$\Delta^{\alpha,\beta}$-γ-Ketohexansäure

(Formel V; R = —CH_3)

Verseifung mit Äthanol und 10%iger KOH. Die in wenig $CHCl_3$ gelöste Säure wurde mit Tierkohle entfärbt, nach dem Filtrieren mit Petroläther versetzt und bei

−17° kristallisiert. Nach mehrfacher Umkrist. Smp. 107–109°. Farblose Kristalle. Ausbeute 20%. Gibt keine $FeCl_3$-Reaktion, keine Fehlingreaktion. Löslich in Äthanol, Aceton; Löslichkeit in H_2O = 0,1%; Äther = 9,5%; Petroläther = 0,01%.

C,H-Bestimmung. $C_6H_8O_3$; Ber.: C = 56,25%; H = 6,25%
Gef.: C = 55,89%; H = 6,35%.

$\Delta^{\alpha,\beta}$-γ-Ketoheptansäure

(Formel V; R = —CH_2—CH_3)

Verseifung mit 4%iger K_2CO_3-Lösung; 10 min erwärmt. Farblose Kristalle; Smp. 111–112°; Ausbeute 15%. Löslichkeit wie oben.

C,H-Bestimmung. $C_7H_{10}O_3$; Ber.: C = 59,15%; H = 7,04%
Gef.: C = 59,14%; H = 7,04%.

$\Delta^{\alpha,\beta}$-γ-Ketooctansäure

(Formel V; R = —CH_2—CH_2—CH_3)

Verseifung mit 4%iger K_2CO_3-Lösung; Farblose Kristalle; Smp. 105–106°. Ausbeute 20%.

C,H-Bestimmung. $C_8H_{12}O_3$; Ber.: C = 61,53%; H = 7,69%
Gef.: C = 62,00%; H = 7,81%.

$\Delta^{\alpha,\beta}$-γ-Keto-ϵ-Methylheptansäure

$$\text{(Formel V; R = —CH} \begin{cases} \diagup \text{CH}_3 \\ \diagdown \text{CH}_3 \end{cases}$$
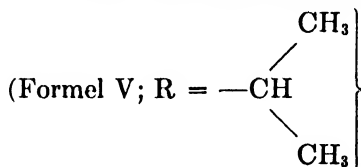
Verseifung mit Äthanol und 10%iger KOH. Farblose Kristalle; Smp. 91–92°. Ausbeute 20%. Löslichkeiten ähnlich wie oben.

C,H-Bestimmung. $C_8H_{12}O_3$; Ber.: C = 61,53%; H = 7,69%
Gef.: C = 61,58%; H = 7,65%.

Der Versuch, Chloral-isobutyliden-keton zu verseifen, führte nur zu öligen dunklen und nicht weiter untersuchten Produkten.

$\Delta^{\alpha,\beta}$-γ-Ketononansäure

(Formel V; R = —$(CH_2)_3$—CH_3)

Durch Verseifung von Chloralamylketon mit Äthanol und 10%iger KOH. Farblose Kristalle; Smp. 117–118°. Ausbeute 40%. Löslichkeit wie oben.

C,H-Bestimmung. $C_9H_{14}O_3$; Ber.: C = 63,53%; H = 8,23%
Gef.: C = 63,38%; H = 8,01%.

$\Delta^{\alpha,\beta}$-γ-Keto-ξ-Methyloctansäure

$$\text{(Formel V; R} = \text{—CH}_2\text{—CH} \underset{\text{CH}_3}{\overset{\text{CH}_3}{\Big\rangle}}$$
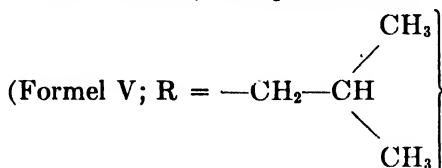
Durch Verseifung von Chloralisoamylketon mit 4%iger K_2CO_3-Lösung. Farblose Kristalle. Smp. 100–103°. Ausbeute 18%.

C,H-Bestimmung. $C_9H_{14}O_3$; Ber.: C = 63,53%; H = 8,23%
Gef.: C = 63,38%; H = 8,01%.

$\Delta^{\alpha,\beta}$-γ-Ketodecansäure

$$\text{(Formel V; —(CH}_2\text{)}_4\text{—CH}_3\text{)}$$

Durch Verseifung von Chloralhexylketon mit 4%iger K_2CO_3-Lösung. Farblose Kristalle. Smp. 110–111°. Ausbeute 50%. Löslichkeit wie oben; in Petroläther etwas leichter löslich als die anderen.

C,H-Bestimmung. $C_{10}H_{16}O_3$; Ber.: C = 65,22%; H = 8,69%
Gef.: C = 65,29%; H = 8,67%.

Semicarbazon der $\Delta^{\alpha,\beta}$-γ-*Ketodecansäure.* Fällt aus Lösung der Säure in Äthanol nach Zugabe einer konzentrierten Lösung von Semicarbazid-chlorhydrat sofort aus. Aus Äthanol umkristallisiert farblose Kristalle vom Smp. 218–219°. N-Bestimmung (Dumas) $C_{11}H_{19}O_3N_3$; Ber. 17,4% N; Gef. 17,2%.

Versuche, das Bis (p-dimethylaminophenyl) ureid nach Breusch und Ulusoy (20) herzustellen, führten wie erwartet in der Lösung zu roten Kristallen, die aber schon nach kurzem weiterem Kochen, anscheinend unter innerer Cyklisierung, in farblose Kristalle vom Smp. 122–125° übergingen; die nicht weiter untersucht wurden.

$\Delta^{\alpha,\beta}$-γ-Ketoundecansäure

$$\text{(Formel V; R} = \text{—(CH}_2\text{)}_5\text{—CH}_3\text{)}$$

Durch Verseifung von Chloralheptylketon mit Äthanol und 10%iger KOH. Farblose Kristalle vom Smp. 120°. Ausbeute 10%.

C,H-Bestimmung. $C_{11}H_{18}O_3$; Ber.: C = 66,66%; H = 9,09%
C = 66,73%; H = 9,18%.

Reduktion der α,β-*ungesättigten* γ-*Ketofettsäuren* (Formel V)

0,5 g. Säure wurde bei den höheren Säuren in 10 ml. Äthanol, bei den niederen in 20 ml. heissem H_2O gelöst, mit 2 g. granuliertem Zink (nicht amalgamiert) und 10 ml. konz. HCl versetzt und das ganze bei Zimmertemperatur 2–3 Stunden durch mechanisches Rühren reduziert; solange bis eine kleine Probe in Gegenwart von überschüssigem K_2CO_3 $KMnO_4$-Lösung nicht mehr sofort reduziert. Dann wurde nach Zugabe von 20 ml. H_2O mehrfach mit Äther extrahiert, der Äther mit Na_2SO_4

getrocknet, filtriert, abgedampft und der kristalline Rückstand aus Benzol und Petroläther umkristallisiert. Ausbeute an γ-Ketofettsäuren (Formel VI) 5–35%.

Zum Beweis der Konstitution der γ-Ketogruppe in den α,β-ungesättigten γ-Keto-fettsäuren wurde zuerst die $\Delta^{\alpha,\beta}$-γ-Ketooctansäure nach der oben beschriebenen Methode in die schon nach einer anderen Methode von Blaise und Koehler (28) synthetisierte γ-Ketooctansäure übergeführt. Beide Säuren vom Smp. 53° erwiesen sich als identisch. Auch die Reduktion der $\Delta^{\alpha,\beta}$-γ-Keto-ε-methylheptansäure zu der von Fittig und de Vos (28a) synthetisierten γ-Keto-ε-methylheptansäure vom Smp. 47° ergab Identität der γ-Ketofettsäuren.

<div align="center">

γ-Ketononansäure

(Formel VI; $R = -(CH_2)_3-CH_3$)

</div>

Aus $\Delta^{\alpha,\beta}$-γ-Ketononansäure durch Reduktion in Äthanol, wie oben angegeben. Weisse Kristalle; Smp. 64–66°. Ausbeute 10%. Reduziert Fehling nicht. Gibt keine $FeCl_3$-Färbung.

<div align="center">

C,H-Bestimmung. $C_9H_{16}O_3$; Ber.: C = 62,97%; H = 9,30%
Gef.: C = 61,89%; H = 8,97%.

</div>

<div align="center">

γ-Keto-ξ-Methyloctansäure

</div>

$$\text{(Formel VI; } R = -CH_2-CH \begin{cases} \diagup CH_3 \\ \diagdown CH_3 \end{cases}$$
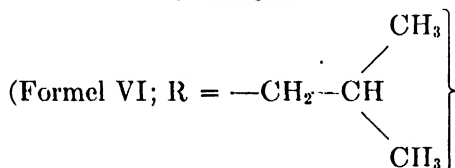
Aus $\Delta^{\alpha,\beta}$-γ-Keto-ξ-methyloctansäure durch Reduktion in wässriger Lösung, wie oben angegeben. Weisse Kristalle; Smp. 50–51°. Ausbeute 20%.

<div align="center">

C,H-Bestimmung. $C_9H_{16}O_3$; Ber.: C = 62,79%; H = 9,30%
Gef.: C = 62,53%; H = 9,09%.

</div>

<div align="center">

γ-Ketodecansäure

(Formel VI; $R = -(CH_2)_4-CH_3$)

</div>

Aus $\Delta^{\alpha,\beta}$-γ-Ketodecansäure durch Reduktion in alkoholischer Lösung; wie oben angegeben. Weisse Kristalle; Smp. 68–69°. Ausbeute 10%.

<div align="center">

C,H-Bestimmung. $C_{10}H_{18}O_3$; Ber.: C = 64,51%; H = 9,67%
Gef.: C = 64,38%; H = 9,61%.

</div>

Oxydation der α,β-ungesättigten γ-Ketofettsäuren (Formel V) zu α,β-Dioxy-γ-ketofettsäuren (Formel VII)

Zur Darstellung wurde nach Fittig (18) und Lapworth, Mottram (19) eine klare 1%ige Lösung der α,β-ungesättigten γ-Ketofettsäuren in K_2CO_3-Lösung bei 10° mit etwa 1,5 Äquivalent einer 1%igen $KMnO_4$-Lösung 5 min. stehen gelassen, dann angesäuert, falls nötig, sofort mit Bisulfit entfärbt und erschöpfend mit Äther

extrahiert. Die abgetrennten und mit Na_2SO_4 getrockneten Ätherextrakte wurden eingedampft und der meist kristalline Rückstand von α,β-Dioxy-γ-ketofettsäuren aus ganz wenig Äthanol + Wasserzugabe umkristallisiert.

α,β-Dioxy-γ-Ketononansäure
(Formel VII; R = —$(CH_2)_3$—CH_3)

Durch Oxydation von $\Delta^{\alpha,\beta}$-γ-Ketononansäure. 15% Ausbeute. Farblose Kristalle. Smp. 120–123°. Reduziert Fehling in der Kälte. Etwa 1% löslich in H_2O. Aus wenig Äthanol + H_2O durch Abkühlen auf −17° umkristallisierbar.

C,H-Bestimmung. $C_9H_{16}O_5$; Ber.: C = 52,94%; H = 7,84%.
Gef.: C = 53,10%; H = 7,94%.

Das *Semicarbazon* der Säure, dargestellt aus der alkoholischen Lösung der Säure und einer konzentrierten wässrigen Lösung von Semicarbazidchlorhydrat in H_2O kristallisiert über Nacht. Smp. 207° unter Zersetzung.

N-Bestimmung. $C_{10}H_{19}N_3O_5$; Ber.: 16,09%N; Gef.: 15,55%N.

α,β-Dioxy-γ-Ketodecansäure
(Formel VII; R = —$(CH_2)_4$—CH_3)

Durch Oxydation von $\Delta^{\alpha,\beta}$-γ-Ketodecansäure. 15% Ausbeute. Löslichkeit in H_2O 0,8%; in Äther 0,7%; in Petroläther unter 0,01%; leichtloslich in Äthanol und Aceton. Smp. 119–120° nach teilweiser vorheriger Erweichung. Reduziert Fehlinglösung schon bei Zimmertemperatur.

C,H-Bestimmung. $C_{10}H_{18}O_5$; Ber.: C = 55,04%; H = 8,25%
Gef.: C = 55,12%; H = 8,41%.

Das *Semicarbazon*, dargestellt wie oben, kristallisiert sofort. Nach Umkristallisation aus Äthanol + H_2O Smp. 199° unter Zersetzung.

C,H und N-Bestimmung. $C_{11}H_{21}O_5N_3$; Ber.: C = 48,00%; H = 7,63%;
N = 15,25%.
Gef.: C = 48,18%; H = 7,33%;
N = 15,07%.

Die C,H-Bestimmungen wurden von Frl. Dr. Bussmann, chemisches Institut der Universität Zürich ausgeführt. Die Schmelzpunkte wurden in einem H_2SO_4-Apparat mit Motorrührung bestimmt und sind nicht korrigiert.

SUMMARY

The following fatty acids have been synthesized in crystalline form: α-hydroxy-γ-keto acids of chain length C_9, C_{10}, C_{11}, C_{13},C_{15}; $\Delta^{\alpha,\beta}$-γ-keto acids C_6, C_7, C_8, C_9, C_{10}; α,β-dihydroxy-γ-keto acids C_9 and C_{10}; besides some branched acids. $\Delta^{\alpha,\beta}$-γ-keto fatty acids give no Fehling reaction, α-hydroxy-γ-keto acids, on warming, and α,β-dihydroxy-γ-

keto fatty acids, in cold solution, give Fehling's reaction. As intermediate products the crystallized 1-trichloro-2-hydroxy-4-keto paraffins of chain length C_6, C_7, C_8, C_9, C_{10}, C_{11} have been synthesized.

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Complexes of Lysozyme

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INTRODUCTION

Although lysozyme has been found to be widely distributed in both animal tissues and in microorganisms, its function in these sources is not particularly clear. It has been demonstrated that lytic action by this enzyme is accompanied by hydrolysis of a mucopolysaccharide in the bacterial membrane (1), but other changes, such as in staining properties, are also observed, and their cause is not evident. It has seemed appropriate, therefore, to investigate complexes between lysozyme and several substances of biological significance.

EXPERIMENTAL

Reagents

The lysozyme was a crystallized sample prepared by Armour and Co. according to the procedure of Alderton and Fevold (2). The other proteins, bovine albumin, pepsin, trypsin, and chymotrypsin were also crystallized products of Armour and Co. The sodium thymus nucleate was a sample sent to Dr. L. Ginger by Dr. J. P. Greenstein. The yeast nucleate and adenylic acid were commercial products obtained from Schwarz Laboratories.

A specially purified sample of sodium dodecyl sulfate was generously supplied by the Fine Chemicals Division of E. I. du Pont de Nemours and Company. The azosulfonamides were obtained through the courtesy of the Winthrop Chemical Company. Methyl orange was a commercial sample of reagent grade.

Procedure

Precipitation tests were carried out by dissolving the lysozyme and other reagent in separate portions of the solvent indicated in Table I, and then mixing the two solutions. The final concentrations are also given in Table I.

TABLE I

Formation of Complexes with Lysozyme[a]

Substance	Final concentration	pH	Buffer	Precipitation[b]
	Per cent			
Sodium thymus nucleate	0.20	4.7	0.13 M Acetate	+
Sodium thymus nucleate	0.20	6.9	0.11 M Phosphate	+
Sodium thymus nucleate	0.20	11.7	0.01 M NaOH	+
Sodium yeast nucleate	0.20	7.6	0.10 M Phosphate	+
Adenylic acid	0.20	7.6	0.10 M Phosphate	0
Bovine serum albumin	0.20	7.6	0.10 M Phosphate	0
Bovine serum albumin	0.20	12.2	0.02 M NaOH	0
Bovine γ-globulin	0.20	7.6	0.10 M Phosphate	0
Pepsin	0.80	4.0	0.09 M Acetate	+
Pepsin	0.80	6.9	0.11 M Phosphate	+
Pepsin	0.80	11.7	0.01 M NaOH	+
Pepsin	0.80	12.2	0.02 M NaOH	0
Trypsin	0.40	4.8	0.13 M Acetate	0
Trypsin	0.40	8.3	0.10 M Bicarbonate	0
Chymotrypsin	0.40	4.8	0.13 M Acetate	0
Chymotrypsin	0.40	8.3 ·	0.10 M Bicarbonate	0
Sodium dodecyl sulfate	0.040	8.3	0.10 M Bicarbonate	+
Sodium dodecyl sulfate	1.00	1.1	0.10 N HCl	+,0
Sodium dodecyl sulfate	1.00	4.0	0.09 M Acetate	+,0
Sodium dodecyl sulfate	1.00	6.9	0.11 M Phosphate	+,0
Sodium dodecyl sulfate	1.00	8.3	0.10 M Bicarbonate	+,0
Sodium dodecyl sulfate	1.00	11.7	0.01 M NaOH	+,0
Sodium dodecyl sulfate	1.00	12.0	0.015 M NaOH	0

[a] Final concentration = 0.20%.

[b] A + sign signifies formation of a precipitate; a 0 sign, no precipitate; +,0, precipitate formed initially disappears as final concentration of reagent is approached.

Dialysis equilibrium studies were carried out by methods described previously (3). With lysozyme, however, it was necessary to establish whether any of this low molecular weight protein (17,500 according to (4)) diffused through the bag. This was accomplished by allowing a 0.2% solution of lysozyme inside the cellophane bag to stand in a tube of phosphate buffer for varying periods. The absorption of ultraviolet light (280 mμ) in the solution outside the bag was then measured, since a previous investigation had indicated that lysozyme has a spectral peak in this region. The results indicate that from 12 to 19% of the protein may diffuse out in a period of 6 hours. This interval of time was a sufficient period, however, for the attainment of equilibrium in the diffusion of the methyl orange anion, as is evident from the data in Table II.

TABLE II

Attainment of Dialysis Equilibrium with Methyl Orange
pH 7, 0°C., 6 hours

Concentration inside bag	Concentration outside bag
$0.355 \times 10^{-5}\ M$	$0.345 \times 10^{-5}\ M$
0.860	0.870

RESULTS AND DISCUSSION

Precipitation Experiments

The substances with which lysozyme was mixed are assembled in Table I, together with details on the properties of the solutions. In general, substances of anionic character have been examined, for they should be most likely to form complexes with lysozyme, as the latter is positively charged up to pH's of about 11 (4).

A general examination of the substances studied shows immediately that not all form insoluble complexes with lysozyme despite the existence of opposite charges in most cases.

Particularly striking, perhaps, is the precipitation of the nucleic acids, both the ribose and desoxyribose types. On the other hand, adenylic acid does not form an insoluble complex with lysozyme. This behavior is analogous to that observed with pteroylglutamic acid conjugase (5) where both yeast and thymus nucleic acid are inhibitors but adenylic acid is not.

Lysozyme does not precipitate bovine serum proteins such as albumin or γ-globulin, despite their anionic character at the basic pH's. It will be impossible to conclude, however, that no complexes are formed until electrophoretic investigations of the solutions are made. Nevertheless, it is evident that opposite charge is not sufficient to insure precipitation of 2 different protein molecules.

Of the proteolytic enzymes investigated, only pepsin was precipitated by lysozyme. This is especially interesting in view of the observation (4) that only pepsin will digest lysozyme which has not been previously heated. Apparently the specificity of biological activity is paralleled by the physicochemical behavior.

Lysozyme is also precipitated by aqueous solutions of sodium dodecyl sulfate, if the latter is present in sufficiently small quantity. At high concentrations, however, the dodecyl sulfate will *redissolve* the

precipitate which is initially formed. These phenomena are analogous to those which have been observed with serum albumin (6).

In contrast to the behavior with serum albumin, the quantity of sodium dodecyl sulfate required to start precipitation with lysozyme is far smaller, on a mole ratio basis. As is evident from Table III, an

TABLE III

Mole Ratios of Dodecyl Sulfate to Lysozyme at pH 8.3[a]

Concentration of protein g./cc.	$\dfrac{\text{Total moles dodecyl sulfate}}{\text{Total moles lysozyme}}$
At onset of precipitation	
0.00474	0.64
0.00475	0.61
0.00479	0.62
Upon redissolving of precipitate	
0.00122	36
0.00228	35
0.00292	39

[a] 0.1 M NaHCO$_3$ used as buffer.

average of less than 1 mole of dodecyl sulfate per mole of lysozyme is sufficient to start precipitation. An estimate from Putnam and Neurath's data (6) on albumin leads to about 10 dodecyl sulfates per molecule of protein at the onset of precipitation. This difference of an order of magnitude is probably due to the relatively few polar carboxyl groups on lysozyme.

The ratios of dodecyl sulfate to protein required to redissolve the insoluble complexes are more nearly comparable. With lysozyme about 36 dodecyl sulfates are present per mole of protein. Since lysozyme contains about 22 cationic groups (7), there are on the average 1.6 sulfates per basic amino acid. With albumin, the data (6) lead to an estimate of about 1.3 for the corresponding ratio. Thus the nature of the soluble complex would seem to be the same in both proteins.

The electrostatic nature of the complexes which are formed seems evident from the effect of pH. At pH's near 12 neither pepsin nor sodium dodecyl sulfate precipitate lysozyme. While the failure of pepsin to form insoluble complexes with lysozyme at high pH's might be attributed to denaturation of the former protein, such an explanation cannot be offered in the case of sodium dodecyl sulfate. It seems apparent, therefore, that precipitation is not obtained in solutions

sufficiently basic to the isoelectric point of lysozyme because the latter loses its positive charge. It is of interest to note in this connection that precipitation by sodium dodecyl sulfate can be obtained with lysozyme for a reasonable distance above the isoelectric point, 10.8 (4), in contrast to the behavior reported for several other proteins (6).

The similarity of organic sulfonates and sulfates in binding by the protein serum albumin (8), suggests that sulfonates of sufficiently high molecular weight might also precipitate lysozyme. This has been found to be the case with orange II (sodium-β-naphthol-p-azobenzenesulfonate) and with azosulfathiazole[1] and azosulfadiazine.[1] An examination of the literature shows similarly that flavianic acid, another naphthalene sulfonate, has been used in early procedures for the isolation of lysozyme (9). It is also of interest to note that the azosulfonamides have been found to suppress the action of lysozyme against *Micrococcus lysodeikticus* (10). Although this antagonistic effect was observed at concentrations of azosulfonamide too low to form a precipitate with lysozyme, it seems most likely that soluble complexes must be present nevertheless.

Dialysis Equilibrium Experiments

An attempt was made to obtain quantitative data on the binding of methyl orange by lysozyme by the dialysis technique (3). Some difficulty was encountered when it was found that a small but significant quantity of lysozyme penetrates the cellophane bag. In the present experiments, however, the leakage of protein may be overlooked, for no significant degree of binding of methyl orange has been observed, as is evident from the data in Table IV. Since it seemed possible that the phosphate buffer might interfere with the binding of the organic anion by a competition effect, one series of

TABLE IV

Absence of Binding of Methyl Orange by Lysozyme[a]
Phosphate buffer (0.1 M), pH 7.6, 0°C.

Conc. of dye in presence of protein	Conc. of dye in absence of protein
$0.37 \times 10^{-5} M$	$0.38 \times 10^{-5} M$
0.94	0.97
1.92	1.96
3.94	3.96

[a] Concentration of 0.200%.

[1] The sulfonamide coupled with acetyl 2-R acid (sodium-1-hydroxy-7-acetylamino-3,6-naphthalene disulfonate).

runs was made in an unbuffered solution containing NaCl to produce the proper ionic strength. No binding was observed in this case either (Table V).

TABLE V

Absence of Binding of Methyl Orange by Lysozyme[a]
Sodium chloride (0.1 M), pH 7.0, 0°C.

Conc. of dye in presence of protein	Conc. of dye in absence of protein
$0.32 \times 10^{-5}\ M$	$0.35 \times 10^{-5}\ M$
0.85	0.87
1.77	1.85
3.48	3.52

[a] Concentration of 0.202%.

In experiments with albumin, unpublished data from this laboratory indicate that sodium dodecyl sulfate has a binding energy at least 2000 cal. greater than that of methyl orange. It is perhaps not too surprising, then, to find no evidence of binding of the latter anion under conditions where the former is bound. From the differences in binding energy with albumin, one would estimate that, if the lysozyme concentration were increased approximately 30-fold, significant quantities of methyl orange would be found. Such experiments will be carried out when sufficient quantities of this protein are available.

ACKNOWLEDGMENT

SUMMARY

Lysozyme has been found to form insoluble complexes with sodium thymus nucleate and sodium yeast nucleate but not with adenylic acid. Among the proteolytic enzymes, pepsin precipitates lysozyme but trypsin and chymotrypsin do not. Similarly the plasma proteins, bovine albumin and γ-globulin do not form insoluble complexes with lysozyme.

From the effect of pH on the precipitation phenomena, particularly with sodium dodecyl sulfate, it is concluded that electrostatic forces contribute strongly to the stability of the complexes. The importance of van der Waals interactions as an additional contribution to the binding energy is emphasized by the failure to detect any significant complexing between lysozyme and methyl orange anions.

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Trace Sugars in Milk

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INTRODUCTION

The published evidence for the occurrence in milk of other sugars than lactose has been rather scanty. However, claims have been made for the alleged presence of glucose (6, 7, 14, 20, 24), arabinose (10, 18), and maltose (20) in cow's milk. Not infrequently these statements have been based on positive reactions with certain color-producing substances, or by fermentation tests (8, 20, 24, 25). Unfortunately, these reactions, unless supplemented by more direct and specific methods, seldom disclose the true chemical nature of the sugar investigated, since they give positive results not only with one sugar, but with a variety of other substances.

In 1908, Sebelien and Kunde (19) crystallized sugar from colostrum and identified it as lactose. Later, by estimating the furfural-forming compounds, they reported the presence of about 50 mg.-% of arabinose in colostrum. Apart from this report, there appears to be no evidence as to the existence of other sugars in colostrum.

Several investigations have been reported concerning the presence of glucose (6, 20), allolactose (15), gynolactose (14), maltose (20), galactose-phosphate ester (6), and free galactose (6) in human milk. From a review of the literature, one is led to conclude that there is a possibility of the existence of other sugars than lactose in milk. However, apart from the investigations of Whitnah and Caulfield (26), the authors have been unable to find any literature pertaining to the isolation and subsequent identification of monosaccharides in milk.

This paper will present the results of experiments designed to isolate and identify any simple sugars which might occur as natural constituents of milk.

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Experimental

1. The First Crystallization Experiment

In this experiment, 10 l. of milk received 2 hrs. after milking was heated to 180°F. for 5 mins., cooled, and then dialyzed in cellophane tubing against 19 l. of distilled water for 20 hrs. The dialyzate was concentrated to 701 g. By fractional crystallization, 7 fractions were isolated, leaving a residual mother liquor which gave a positive reaction for monosaccharide by a modified Barfoed test (23). All attempts to induce crystallization of sugars from this syrup failed. This was attributed to the presence of non-sugar constituents of the whey, which inhibited the crystallization of lactose. Further, even if the lactose could be crystallized from this syrup, the subsequent isolation of monosaccharides would be exceedingly difficult unless most of the foreign material could be removed. Hence, it was proposed to study means of removing the non-sugar impurities prior to crystallization without affecting the lactose and other sugars in any way.

It seemed that the synthetic ion-exchangers should be effective in removing the non-sugar constituents from the milk-dialyzate, and that complete crystallization of the sugars present would then become possible.

2. The Second Crystallization Experiment

Ten l. of milk received within 2 hrs. after milking was heated immediately to 180°F. for 5 mins., and cooled to room temperature in ice water. This sample was dialyzed as before against 16 l. of distilled water. Formalin was used as a preservative. The dialyzed milk was redialyzed against another 16 l. of water. The two dialyzates, containing 84.4% of the total sugar in the milk, were combined and passed through the cation exchanger (IR-100) and then through the anion absorber (IR-4B). The synthetic resins were obtained from Resinous Products and Chemical Company, Inc., Philadelphia, Pa. Amberlite IR-100, sold in sodium form, was converted to its hydrogen derivative before use, by treatment with 5% hydrochloric acid. The anion exchanger IR-4B were converted to the free amine form with 2% sodium carbonate. Both exchanger columns were washed with 12 l. of water, which was added to the original demineralized dialyzate, making a total volume of 45 l.

The salt-free sugar solution was concentrated repeatedly, and by successive crystallizations 10 fractions, X_1 to X_{10}, were obtained. Each of the fractions was washed first with ice-cold water, and then with ice-cold alcohol. All of the fractions were crystalline, the first 7 being white in color and the others yellowish-brown. The crystallization of the last 3 fractions was very slow and was induced by very careful seeding.

The total reducing value of each of the fractions was determined by a modification of Somogyi's colorimetric method (21) adapted for the determination of lactose, and each was tested for the presence of monosaccharide by means of Tauber and Kleiner's (23) reagent, using 1.00 mg. of each of the samples. The results are presented in Table I.

TABLE I

The Weight and Apparent Lactose Content of Fractions X_1 to X_{10}

Sample no.	Yield	Lactose	Barfoed's test for monosaccharides
	g.	*per cent*	
X_1	114.0	96.7	—
X_2	80.0	98.7	—
X_3	13.0	96.7	—
X_4	36.0	94.0	—
X_5	30.0	94.0	—
X_6	3.0	94.0	—
X_7	10.5	92.7	—
X_8	0.60	94.2	$+$
X_9	0.52	110.0	$+$
X_{10}	0.40	133.3	$+$

If it is assumed that all the fractions obtained from the dialyzate contained only lactose, the higher reduction values of samples X_9 and X_{10} cannot be easily explained. They can be explained by assuming that these fractions contain hexoses or other substances with a greater reducing power than that of lactose.

3. Identification of the Unknown Sugar in the Fractions X_8–X_{10}

Since these fractions yielded a positive reaction for monosaccharides, it was perhaps logical to suspect the presence of one or more naturally occurring monoses. With mixtures, it is usually necessary to resort to combined methods for the analysis. Selective fermentation with specific organisms have been successfully employed by a number of investigators for the estimation of individual sugars. Bellamy, Doob and Gunsalus (unpublished) developed strains of *Streptococci* which could be adapted to ferment glucose or galactose selectively. They devised a resting-cell technique for the differential fermentation of glucose and galactose by *Streptococci* 68C and G_2 (department numbers), respectively. This procedure was found to be quite satisfactory and we adopted it in this investigation for the identification and estimation of the unknown sugars in some of the fractions.

Two ml. of cell suspension was added to each of the centrifuge tubes containing 10 ml. of sugar solution and incubated for 45 mins. at 37°C. Each tube contained 2.00 mg. of sugar. Pure samples of glucose, lactose, and the fractions X_8–X_{10} were taken for the experiment. A tube containing 10 ml. of water and 2 ml. of cell suspension served as control. After the incubation, the suspensions were placed in ice water for

10 mins., centrifuged, and the supernatants decanted. Aliquots from this were used for the determinations of total reducing value by Somogyi's colorimetric method (21) and of lactic acid by the method of Barker and Summerson (1). The results are presented in Table II.

TABLE II

Results of Fermentation Tests Using Streptococci 68C

Kind of sugar	Sugar taken for expt.	Sugar after fermentation	Sugar used up	Lactic acid produced	
				Observed − blank	Corrected
	mg.	*mg.*	*mg.*	*mg.*	*mg.*
Glucose	2.00	0.036	1.964	1.903	1.964
Lactose	2.00	2.004	—	—	—
Galactose	2.00	1.992	0.008	—	—
X_8	2.00	1.956	0.044	0.013	0.014
X_9	2.00	1.668	0.332	0.312	0.322
X_{10}	2.00	0.672	1.328	1.243	1.283

Note: The correction for the lactic acid in the last column was found necessary because of the fact that the lactic acid produced did not correspond to the sugar decomposed. Therefore, calculating the percentage of conversion for glucose, similar corrections were made for all fractions.

Except for glucose and galactose all the other reducing values were calculated as lactose, and all the results were corrected for the blank. It is clear from the table that the organism is specific for glucose and had no action on galactose or lactose.

Although the above experiment indicated the existence of glucose in fractions X_8–X_{10}, there was no evidence either for or against the presence of galactose. To obtain such evidence, the resting cell method of analysis as described for *Streptococci* 68C was repeated using *Streptococci* G_2. This experiment indicated that the fractions X_9 and X_{10} contained no measurable amount of galactose, and that the fraction X_8 was practically pure lactose.

From the resting cell experiments it was found that fractions X_9 and X_{10} contained glucose to the extent of 16.5 and 77.4%, respectively. Since the yields of these fractions were known, the total glucose content can be calculated. This amounted to 0.351 g. and originated from the dialyzate containing 84.4% of the total sugar of milk. Hence, assuming that equilibrium had been reached in the dialysis, the glucose content of the original milk amounted to about 4 mg./100 ml.

Having obtained positive evidence that some of the fractions contained glucose, the problem of isolating this sugar became of importance. It was found that glucose is much more soluble in alcohol than lactose and it was thought that this property might be used in separating the sugars. It was also found that glucose could be extracted from a mixture of glucose and lactose by cold alcohol, from which the glucose could be crystallized in a fairly pure state.

Fractions X_9 and X_{10} were mixed together and extracted thrice with alcohol, at room temperature, using 50 ml. the first and second time, and 25 ml. in the third extraction. The combined extracts were concentrated to a small volume and allowed to crystallize. The crystals were separated, washed with ice-cold alcohol and recrystallized thrice from alcohol. The crystallization was very slow and the final yield of the crystals X_{14} was 0.280 g., which assayed 99.9% glucose when tested by the colorimetric method (21).

This fraction, X_{14}, is believed to be the first preparation of crystalline glucose ever isolated from normal milk.

4. Third Crystallization Experiment

To confirm the results obtained in the second crystallization studies, the experiment was repeated. From 18 l. of milk a fraction L_{22}, weighing 0.503 g., was obtained. This assayed 99.9% glucose when tested colorimetrically (21). On the basis of these experiments, assuming that dialysis-equilibrium had been attained, the concentration of glucose amounts to 5 mg./100 ml. of milk.

5. Further Evidence on the Nature of the Sugar Isolated from Milk

A few more experiments were conducted to determine a few of the physicochemical characteristics of the sugar and its derivative and the results are presented in the following pages.

The purity of the crystalline fractions X_{14} and L_{22} was tested by the copper-iodimetric method (21) and they gave values as glucose 99.35 and 99.45%, respectively.

The specific rotation of L_{22} was initial $[\alpha]_D^{25} = +100°$ and final $[\alpha]_D^{25} = +51.7$. Ten mg. of the fraction L_{22} formed crystalline needle-shaped glucosazone which appeared in 14.9 mins., while a control glucose sample gave the osazone in 14.25 mins. A photomicrograph of the osazone from L_{22} is shown in Fig. 1. The osazone was prepared from 100 mg. of L_{22} and it melted at 202°C. A mixed melting point determined with an authentic specimen caused no depression. The osazone gave in pyridine-

alcohol mixture (4.6) an initial $[\alpha]_D - 62°$ and final $[\alpha]_D - 34°$; and a value of 15.4% for nitrogen as against 15.64% theoretical.

Fractions X_{14} and L_{22} were tested for fructose both by resorcinol (9) and diphenylamine (2), using aliquots containing 0.10 mg., and were found to give negative results.

Fig. 1. A photomicrograph of the osazone prepared from fraction L_{22}.

6. Identification of Glucose as Osazone from Milk Serum

Since non-sugar constituents such as urea interfere with the osazone test (5), the sugars from 200 ml. of deproteinized, de-ionized milk were separated first from the non-sugar impurities by applying Salkowski's (17) copper-lime method. The sugars recovered from the copper-lime precipitate were separated by a chromatographic technique (11).

The adsorbent used, Florex XXX, was a commercial product suppled by Floridin Company, Warren, Pa. It was used in admixture with a filter-aid (Celite No. 535). The adsorbent was packed properly in a tube of 2.5 cm. diameter and 18 cm. height, and the sugar-mixture, dissolved in a small quantity of hot water, was passed through the column under suction. The chromatogram was developed with 150 ml. of 95% alcohol. The column was extruded and streaked with alkaline permanganate. We were able to mark only 2 zones. The glucose zone was observed 6 cm. from the bottom, having a width of 1.5 cm. The portions containing the respective sugars were repacked in chromatographic tubes and eluted with 85% alcohol. The eluates were concentrated to dryness and the osazones were prepared. They were identified as glucose and lactose by their crystalline shapes. The osazone from glucose appeared while hot and there was no indication of lactose in this eluate.

The experiment was repeated with 100 ml. of a sample of first day's colostrum from a Jersey cow. Here again only 2 bands were observed and we were able to identify only glucose as a monose.

7. Determination of Glucose in Milk

After having established the existence of glucose in normal milk the problem of devising a method for the determination of glucose in milk became important. A few experiments were conducted to study the separation of glucose from lactose.

The de-ionized filtrate from 200 ml. of milk was concentrated to a small volume and most of the lactose was crystallized out. The mother liquor and washings were concentrated to dryness and a series of cold extractions and estimations of monosaccharide by Tauber and Kleiner's method (23) showed that at least 6 extractions were necessary. On this basis the glucose content of this sample of milk was found to be 6.87 mg./100 ml. of milk. Since such extractions are cumbersome, it was proposed to absorb mixtures of glucose and lactose solutions on filter paper strips and then extract the dried filter paper with alcohol in a Soxhlet apparatus. In a few trials it was found that 5 extractions were necessary to recover all monoses in the extract. These experiments were extended to milk and the procedure is as follows.

One hundred ml. of milk was diluted to 800 ml. and the proteins were precipitated with barium hydroxide and zinc sulfate (21). The filtrate was de-ionized, concentrated to about 10 ml., absorbed on filter paper strips and dried at 100°C. for 3 hrs. The filter paper was then extracted 5 times in a Soxhlet apparatus with aldehyde-

TABLE III

Glucose Content of Milk

(Results expressed in mg./100 ml. of milk)

Sample no.	Alcohol-extractable Lactose	Glucose
1	69.0	6.00
2	66.0	5.65
3	34.2	6.60
4	53.5	5.85
5	27.0	5.90
6	26.0	4.08
7	31.8	5.20
7a	50.0	10.40
8	43.5	7.58
9	37.8	5.50
10	25.2	5.90

free alcohol. The extract was concentrated to about 5 ml. and allowed to crystallize. The crystals of lactose were filtered out, washed twice with alcohol and the mother liquor and washings were concentrated to dryness. The residue was dissolved in water,

made to a known volume, and aliquots from this were used for estimating lactose (21) and glucose (23).

The results of the analysis of fresh evening samples from individual cows are presented in Table III.

Sample 7a was the same as 7 but contained 5.00 mg. of added glucose. All glucose determinations were made on aliquots of solution containing not more than 2.0 mg. of sugar calculated as lactose. Samples 3, 5, 7, and 10, tested with Dische's reagent (4), gave a greenish blue color indicating glucose. Pentoses under the same test give a pink coloration.

The results of the analysis of some samples of colostrum are presented in Table IV.

TABLE IV

Glucose Content of Colostrum

(Results expressed as mg./100 ml. of colostrum)

Sample no.	Breed	Hours after calving	Alcohol extractable	
			Lactose	Glucose
1	Holstein	12	22.0	15.00
2	Holstein	36	22.4	10.75
3	Holstein	60	20.6	5.95
4	Holstein	72	22.0	3.10
5	Holstein	96	19.4	3.18
6	Holstein	120	24.0	3.75
7	Holstein	240	47.6	5.70
8	Jersey	1	29.0	12.50
9	Jersey	24	16.6	9.00
10	Jersey	240	44.0	6.81

Samples 1 and 8, when tested for pentose, gave a negative result. The concentration of glucose in the normal milk samples analyzed in our laboratories never exceeded 8 mg./100 ml. of milk. In one trial we used *Streptococci* 68C on a de-ionized serum from 2 ml. of the milk, and the net amount of lactic acid produced only confirmed our value for glucose. Since we were interested in a chemical method, the latter trials were not pursued further.

Discussion

Experiments with pure sugars showed that free glucose and galactose diffused readily through the membrane and equilibrium was established within 4 hrs. This would not be true of sugars combined with colloidal material. Sorensen (12, 22), in an extensive investigation of the crystallization of proteins from whey, reported that purified crystalline lactalbumin contains galactose and mannose. This material had been subjected to dialysis during purification. If glucose were present as a hexose phosphate it would be retained by the ion exchange resins (13). It seems reasonable to assume that the glucose recovered in these experiments is either free in the milk or exists in a very loose state of combination with the protein.

A second question may be raised. Is the glucose present in milk in the free state at the time of milking or is it derived from lactose by hydrolysis? The fact that the milk was heated immediately after milking to destroy enzymes and bacteria, and that the dialyzate and milk were preserved with formalin, should reduce the likelihood of enzymatic action.

It is true that the lactose was subjected to a pH as low as 2 when it passed through the cation exchanger. However, experiments with pure lactose solution gave no evidence of hydrolysis under these conditions. Furthermore, if the glucose were derived from lactose, then an equivalent quantity of galactose should be present, but no galactose could be found. It seems unlikely that the glucose found in milk was derived from the lactose.

That the fractions X_{14} and L_{22} isolated from milk assayed over 99.0% purity was established by both colorimetric and copper-iodimetric methods. All the tests that we have used confirmed that these samples behaved in a way similar to pure glucose.

Most of the earlier workers (8, 20, 24) reported yeast-fermentable sugar of milk as glucose, and the values we have obtained are considerably lower than the values reported by these workers. By pumping glucose into the stomach of lactating cows, Cave and co-workers (3) found significant increase in the yeast-fermentable sugar of milk. On the contrary, Polonovski (16) observed that the addition of 100–200 g. of glucose to the diet of women caused no change in milk glucose. Hence, it is difficult to presume that feed has any effect.

Since glucose is a normal constituent of blood, it is not surprising

that it is present in milk also. Milk is known to contain traces of many blood constituents. Their presence may or may not be of importance.

SUMMARY

1. Details of the fractional crystallization experiments, using dialyzed, de-ionized milk serum, are reported. From the last few fractions, by alcohol extraction and crystallization, a simple sugar was for the first time isolated in crystalline form from milk.

2. This sugar was identified as glucose by its reducing value, optical rotation, crystalline form, melting point, the mutarotation of the osazone, by specific fermentation tests, and by a few color reactions.

3. By using Salkowski's copper-lime method the sugars from de-ionized milk were separated from non-sugar constituents and from the regenerated sugar solution, the sugars were separated by a chromatographic method. These sugars were identified as lactose and glucose by osazone tests.

4. Glucose accounted for the whole of the monoses present in milk. Little, if any, other free sugar exists in milk.

5. A chemical method was devised for the determination of glucose in milk. The level of glucose in milk was found to vary between 4.08 and 7.58 mg./100 ml. of milk.

6. The analysis of a few samples of colostrum revealed that they contain much more glucose than normal milk. There was a gradual decrease in the glucose content of colostrum in the days following parturition. The normal value was reached after 10 days.

7. It was concluded that the glucose exists in milk in the free state, and probably arises from the blood of the cow.

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The Effect of B Vitamin Deficiencies on Tryptophan Metabolism in the Rat[1]

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INTRODUCTION

It has been repeatedly demonstrated that pyridoxine-deficient animals excrete larger quantities of the tryptophan metabolites, xanthurenic acid and kynurenine, than normal animals (1–4). On the other hand, kynurenic acid, which is excreted by both normal and deficient animals, has been considered a product of the normal metabolism of tryptophan (5).

Since the quantitative relationships among the various tryptophan metabolites are obscure, and since other vitamins of the B complex have also been implicated in amino acid metabolism, it was considered of interest to study quantitatively the effects of deficiencies in certain B vitamins upon tryptophan metabolism.

EXPERIMENTAL

The procedure was similar to that recently described (4). Adult male albino rats were fed purified diets free of thiamine, riboflavin or pyridoxine until their weights had become static or were decreasing. From 2 to 5 weeks were required to reach this state, depending upon the vitamin deficiency involved. Mild deficiency states were employed to minimize general ill health and secondary deficiencies due to inanition.

After the depletion period, groups of 3 rats weighing 180–210 g. each were dosed by stomach tube with L-tryptophan (230 mg.) and the B vitamins (1 mg. each) in which they were not deficient. After 19.5 hours each rat received 10 cc. of water by stomach tube and urines were collected with cage washings 3 hours later.

Analytical Methods

The methods for the determination of kynurenine and xanthurenic acid [2] have been described (4). Kynurenic acid was determined as follows: [3] Ten cc. of urine was acidified to Congo red with 3 N HCl and stored in the cold overnight. The precipitate was collected, washed once with 3 cc. of cold 0.3 N HCl, then dissolved in 0.5 cc. of 5 N NaOH, and diluted to 10 cc. with water. An aliquot of this solution, estimated to contain between 0.05 and 0.5 mg. of kynurenic acid, was diluted to 10 cc. with 0.25 N NaOH. The solution was heated in a vigorously boiling water bath for 15 minutes, cooled, treated with two 1 cc. portions of 30% H_2O_2, each addition being followed by 30 mins. heating and then cooling. Finally, 2 cc. of 3 N HCl were added and the volume of the solution was adjusted to 10 cc. with water. One cc. each of 0.1% $NaNO_2$, 0.5% ammonium sulfamate, and 1.0% N-(1-naphthyl)-ethylenediamine dihydrochloride were added, at 3 minute intervals, with shaking. After one hour the intensity of color developed was compared at $\lambda = 555$ mμ with that of known quantities of kynurenic acid carried through the same process.

This method is not quantitative if the urine contains less than 0.2 mg. of kynurenic acid/cc., since at lower concentrations precipitation may be less than 60% complete, and recoveries from urine and from water are divergent.

Of the known substances which might interfere with the determination, tryptophan, indoleacetic acid, and kynurenine are not precipitated, and xanthurenic acid, although precipitated, gives no color with the reagents after treatment with alkaline hydrogen peroxide. If other quinoline derivatives are formed from tryptophan in the rat (7), and are precipitated from the acidified urine, they might also be determined by the above method.

RESULTS

Normal rats excreted about 17% of the administered tryptophan as kynurenine, kynurenic acid, and xanthurenic acid. The two quinaldic acids were excreted in approximately equal amounts, while kynurenine excretion was lower.

Rats mildly depleted of thiamine excreted about the same fraction of administered tryptophan in the form of the metabolites. The excretion pattern was altered, however, in such a way that a smaller proportion of the amino acid was excreted as kynurenic acid, and a larger proportion as xanthurenic acid.

[2] Dr. H. P. Sarett, in a personal communication, has stated that, in preparing the ferric chloride reagent for the estimation of xanthurenic acid, the treatment with bicarbonate is unnecessary. We have checked this finding. The intensity of color produced is exactly comparable to that produced by the bicarbonate-treated reagent.

[3] Kretschy (6) obtained anthranilic acid by the alkaline permanganate oxidation of kynurenic acid, followed by hydrolysis. As an analytical reagent, H_2O_2 appears to offer certain advantages over permanganate. The procedure described yields about 10% of the theoretical amount of anthranilic acid as judged by its color reaction.

The riboflavin-deficient rats excreted a larger proportion of administered tryptophan as the 3 metabolites, with relatively more kynurenic acid and less xanthurenic acid appearing in the urine.

The pyridoxine-deficient rats excreted a much larger proportion of the tryptophan as identified metabolites, and the excretion pattern was shifted in favor of xanthurenic acid at the expense of kynurenic acid.

The multiple vitamin deficiencies produced changes in agreement with the above. Whereas thiamin deficiency shifted the pattern to favor the excretion of xanthurenic acid, and riboflavin deficiency shifted it to favor the excretion of kynurenic acid, in the rats deficient in both, an essentially normal pattern was produced. Again, riboflavin deficiency caused a decreased excretion of xanthurenic acid, whether or not the animals were also deficient in pyridoxine.

In an additional experiment, it appeared that the administration of galactoflavin[4] (1 mg. per rat) did not add to the effect of riboflavin deficiency. However, the n-butyl analogue of thiamine aggravated the effect of thiamine deficiency, and pyridoxine-deficient rats which received 4-desoxypyridoxine excreted the total dose of tryptophan as kynurenic acid, xanthurenic acid, and kynurenine.

DISCUSSION

It has been shown that in animals deficient in riboflavin or pyridoxine, the recovery of L-tryptophan as known urinary metabolites is increased, and it may be inferred that these deficiencies inhibit the conversion of L-tryptophan to unidentified products. The formation of unidentified products appears to proceed principally directly from L-tryptophan, but the pathway through kynurenine may be involved to some extent.

An increase in the excretion of either kynurenic acid or xanthurenic acid without a proportionate increase in the other is taken as evidence that the formation of one of the two quinaldic acids from kynurenine is proceeding at a suboptimal rate. Hence, the observation that disproportionate increases in xanthurenic acid excretion are produced in thiamin and in pyridoxine deficiency indicates interference with the conversion of kynurenine to kynurenic acid. On the other hand, the

disporportionate increase in kynurenic acid excretion by riboflavin-deficient rats suggests that the conversion of kynurenine to xanthurenic acid is hindered by this deficiency.

The finding that the excretion of kynurenic acid can be increased by a vitamin deficiency renders ambiguous the statement that kynurenic acid is a normal metabolite of tryptophan. It is possible that rats receiving ample vitamins and minimal quantities of tryptophan would excrete essentially none of the metabolites under scrutiny.

TABLE I

The Excretion of Metabolites by Rats Dosed with L-Tryptophan

| Diet | No. of groups | Metabolite excreted compared with the sum Xa+Ka+Ky | | | Tryptophan excreted as Ka, Xa, and Ky |
		Ka[a]	Xa	Ky	
		per cent	*per cent*	*per cent*	*per cent*
Complete	5	41 ± 1.0^b	39 ± 2.3	20 ± 2.3	17 ± 2.4
B_1 Deficient	3	22 ± 2.3	61 ± 0.6	16 ± 2.5	25 ± 7.9
B_2 Deficient	4	69 ± 6.5	20 ± 5.6	11 ± 1.4	43 ± 5.0
B_6 Deficient	4	11 ± 1.8	64 ± 1.8	25 ± 3.5	73 ± 9.8
B_1, B_2 Deficient	3	53 ± 6.1	37 ± 5.4	10 ± 1.1	37 ± 4.9
B_1, B_6 Deficient	3	13 ± 1.0	64 ± 3.7	23 ± 2.7	80 ± 8.0
B_2, B_6 Deficient	3	23 ± 0.4	47 ± 4.2	30 ± 4.1	72 ± 9.6
B_1, B_2, B_6 Deficient	3	21 ± 0.4	63 ± 2.8	20 ± 0.7	76 ± 18.2

[a] Ka = Kynurenic acid; Xa = Xanthurenic acid; Ky = Kynurenine.
[b] Standard error.

SUMMARY

The effects of mild B vitamin deficiencies on the fate of L-tryptophan in the rat are as follows:

(1) In riboflavin-deficient rats the administration of tryptophan results in a 2–3-fold increase in the excretion of xanthurenic acid, kynurenic acid and kynurenine over that noted in normal animals. The excretion of kynurenic acid is disproportionately large.

(2) In thiamine-deficient rats, the urinary excretion pattern of the 3 metabolites is shifted in favor of xanthurenic acid, but there is no increase in the fraction of tryptophan excreted as the 3 metabolites.

(3) Pyridoxine deficiency increases the excretion of xanthurenic acid 7-fold, and of kynurenine 5-fold, while the excretion of kynurenic acid is not altered.

(4) Riboflavin deficiency superimposed upon either pyridoxine or thiamine deficiency reduces the excretion of xanthurenic acid and increases that of kynurenic acid. The effects of deficiencies in thiamine and pyridoxine are not additive.

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The Photolysis of Cotton Cellulose. I. Production of CO₂ on Irradiation of Cellulose with Intense Ultraviolet Light

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The tenderizing of cotton fabrics on their exposure to sunlight has been observed by practically everybody who has had anything to do with these fabrics. It has long since been established that the degradation which occurs is induced by the ultraviolet region of the solar spectrum. The tenderizing effect which has been observed has been correlated [1,2] with the decrease in the viscosity of cellulose solutions in cupriammonium hydroxide, by an increase in the copper number, by an increase in the degree of acidity of the cellulose, a decrease in the methylene blue number, and an increase in the silver number. With respect to the changes which occur, it has been assumed that the product from the ultraviolet light irradiation is oxycellulose,[2] and the report is that the loss of strength of cellulose fabrics does not occur in the absence of oxygen.[1] Carbon dioxide, carbon monoxide, and possibly hydrogen, have been found as degradation products.[1,3] The loss in strength is accelerated by the presence of water vapor.[2]

It is reported [3] that the photochemical effect of ultraviolet light predisposes cellulose to oxidation; thus, with cellulose which has been exposed to ultraviolet irradiation in a nitrogen atmosphere, a decrease in the viscosity of the cupriammonium hydroxide solution of the cellulose occurs, provided only that the cellulose is subsequently exposed to air. There was no apparent change in the copper number of the α-cellulose on irradiation until the cellulose was exposed to oxygen, whereupon the copper number increased and the α-cellulose decreased.[3] The conclusion reached,[3] on the basis of viscosity data, was that the degree of polymerization of the cellulose is reduced by the combined effect of the irradiation and atmospheric oxygen.

Cellulose is effectively degraded by the 360–70 mμ region from the mercury vapor lamp, as was conclusively demonstrated by Wagner, Webber and Siu.[4] Although they did not establish that other lines in the mercury vapor spectrum are not effective, the reduction of the Pressley index[5] with the 360–70 mμ region was as great as with the entire spectrum from the mercury vapor arc.

[1] Barr, Guy, and Hadfield, I. H., *J. Textile Inst.* **18T**, 490 (1927).
[2] Cunliffe, P. W., Farrow, F. D., and Medgley, E., *ibid.* **19T**, 169 (1928).
[3] Stillings, R. A., and Van Nostrand, R. J., *J. Am. Chem. Soc.* **66**, 753 (1944).
[4] Wagner, R. P., Webber, H. H., and Siu, R. G., *Arch. Biochem.* **12**, 37 (1947).
[5] Pressley, E. H., *Am. Soc. Testing Materials, Bull.* **118**, 13 (1942).

In the experiments reported herewith we have been able to demonstrate the production of carbon dioxide on ultraviolet irradiation of dewaxed cotton in the absence of any demonstrable quantity of oxygen. The evidence presented herewith for the production of carbon monoxide and hydrogen is negative. We have been able to confirm the post-irradiation effect in that we have demonstrated the production of carbon dioxide after the ultraviolet lights have been turned off, providing a stream of air is passed over the cellulose.

Absorption Spectrum of Cellulose

Relative to the determination of the absorption spectrum with cellulose in the region 300–400 mμ, difficulties of considerable magni-
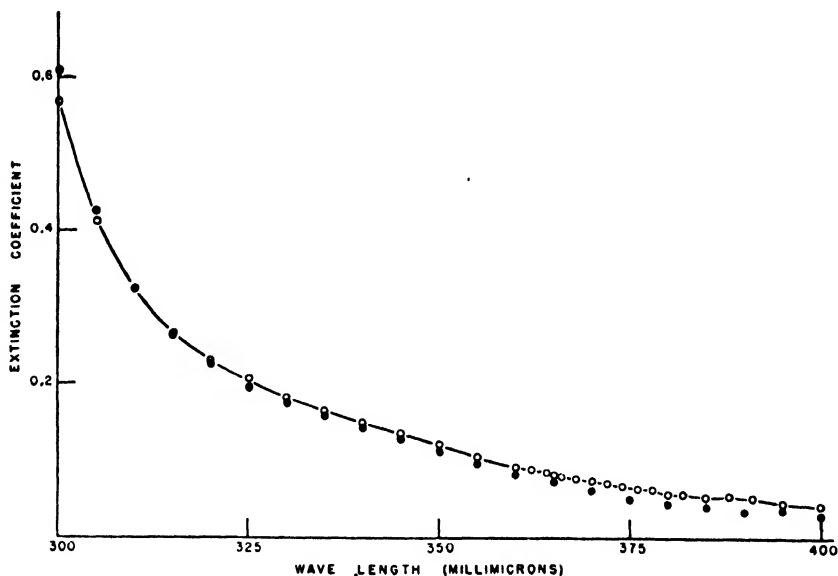
Fig. 1. Absorption curve for cupriethylenediamine (●) and for a solution of cellulose in cupriethylenediamine (○).

tude are encountered with the cotton fiber. Objections may be raised to the determination of the absorption spectrum of cellulose in solution, and perhaps conclusions reached as a result of such observations may not be completely valid. Cellulose in solution, however, is modified on irradiation with ultraviolet light. The viscosities of oxygen-free cellulose solutions (0.05%) in cupriethylenediamine in corex viscosim-

eters were reduced 2.3–12% (5 determinations) on irradiation for 4 hours with 2 Hanovia S C 5010 analytical model quartz mercury vapor lamps. (Although the intensity of the light was not determined in these experiments, it was established by Wagner, Webber and Siu[4] that, with the new lamps, the intensity in the region 250–390 mμ was approximately 100 times that in summer sunlight.)

We determined the absorption spectrum in the region 300–400 mμ for solutions of cotton cellulose in cupriethylenediamine. The Beckman Spectrophotometer was used. Data are presented in Fig. 1 for a 0.05% cellulose solution in 0.01 N cupriethylenediamine, together with the data for the solvent. It will be noted that the curves are very similar; the data do not indicate any absorption maximum for cellulose in the region investigated. There appears, however, to be slight, but continuous, absorption by the cellulose in the region 370–400 mμ. Irradiation of the cellulose in solution did not noticeably alter the absorption spectrum.

Production of Carbon Dioxide in an Oxygen-Free Atmosphere

The apparatus used in this work, on the irradiation of cotton cellulose fibers is represented diagrammatically in Fig. 2. The dry dewaxed cotton (dewaxed with
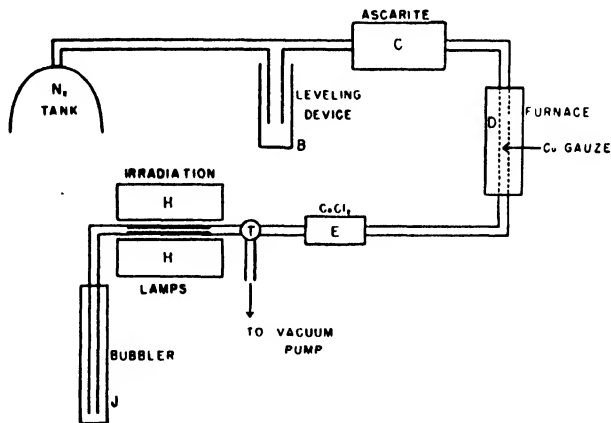
FIG. 2. Apparatus used in the ultraviolet treatment of cotton cellulose.

ethyl alcohol in a Soxhlet apparatus) was irradiated in a water-cooled corex irradiation chamber which was placed in the train between the two Hanovia ultraviolet lamps at a distance of approximately 22 cm. from the burners. Precautions were taken to secure preparations that were oxygen-free. It was established that the evacuation of the irradiation chamber with a duo-seal (Welch) vacuum pump and flushing with oxygen-free nitrogen 8 successive times was sufficient to remove all detectable quantities of oxygen from the gas phase in the apparatus. The stream of nitrogen from the nitrogen tank was rendered free of any detectable quantity of oxygen (alkaline

pyrogallol) by passing the stream over copper gauze heated to a dull red heat in a combustion furnace. Carbon dioxide was removed by passing the gas stream through ascarite. The stream was then passed through a calcium chloride tube before it entered the irradiation chamber. Carbon dioxide was trapped by passing the stream from the irradiation chamber through a 230 cm. tower of 0.01 N carbon dioxide-free sodium hydroxide solution. Complete absorption of the carbon dioxide was assured by breaking the gas stream up into extremely small bubbles. In the determination of the change in strength of the sodium hydroxide solution, barium chloride was added, and after the barium carbonate had settled, aliquots were titrated with 0.01 N hydrochloric acid. No detectable quantities of carbon dioxide were recovered in the absence of ultraviolet irradiation.

The time of irradiation of the fibers in each case was 16 hours, during which time a continuous stream of nitrogen was passed through the apparatus. The data presented in Table I are typical of those (21

TABLE I

Quantity of Carbon Dioxide Produced on the Irradiation of Dewaxed Cotton Fibers for 19 Hours with High Intesity Ultraviolet Light

Experiment	Cellulose	CO_2 produced	$mM.$ CO_2/g. cellulose
	g.	*mM*	
A	0.5	0.085	0.170
B	0.5	0.073	0.146
C	0.5	0.060	0.120

experiments) we have obtained. From 0.0012 to 0.009 millimols of CO_2/g. of cellulose were reported by Stillings[3] *et al.* to be produced after 24–336 hours of exposure. The differences are probably due to differences in intensity of ultraviolet light used.

Determination of Carbon Monoxide

It was found that reliable results could not be obtained by measuring simultaneously the quantities of carbon dioxide, carbon monoxide, and hydrogen which might have resulted from the irradiation experiments. It was expedient to make separate determinations. With reference to the determination of carbon monoxide, the train was modified in that the stream of gas coming from the irradiation chamber was passed through ascarite for the purpose of removing carbon dioxide, and then it was passed over cupric oxide granules which were heated to a dull red in a combustion furnace. The gas stream was then led through the sodium hydroxide column as indicated above, and the quantity of carbon dioxide collected was determined by means of titration. The decrease in normality of the sodium hydroxide in each of five experi-

ments was scarcely perceptible, and such as may have occurred could have resulted from the failure of the ascarite to quantitatively remove the carbon dioxide. This evidence, with respect to the carbon monoxide, is not in keeping with the reports by Cunliffe, Farrow and Medgley,[2] by Barr and Hadfield,[1] and by Stillings and Van Nostrand,[3] who found quantities of carbon monoxide comparable with quantities of carbon dioxide produced in their experiments.

By way of confirmation of the results which we have obtained, the stream of gas coming from the irradiation chamber was passed through citrated bovine blood at a dilution of 1:100, and the absorption
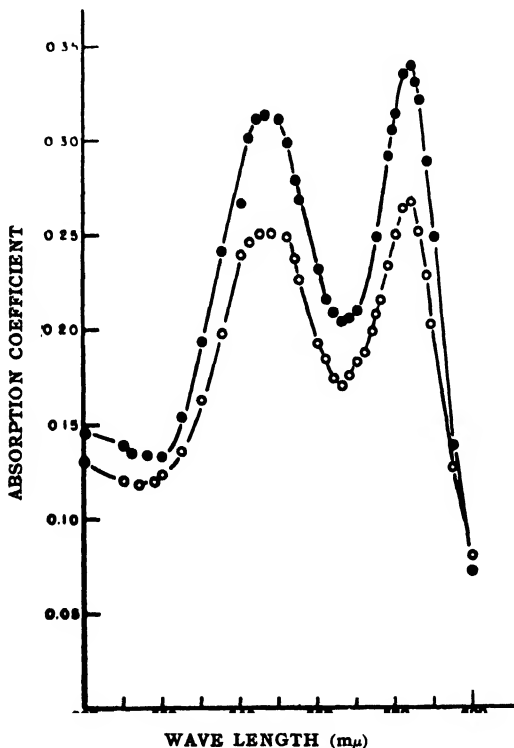
FIG. 3. Absorption spectra of oxyhemoglobin. ● original preparation;
○ preparation exposed to gases from the irradiation chamber.

spectrum of the preparation was then determined with the Beckman Spectrophotometer after 16 hours irradiation. The absorption spectrum for the original oxyhemoglobin, and for the oxyhemoglobin which had been exposed to the gases from the irradiation chamber, are presented in Fig. 3. The evidence would indicate that there is no measur-

able quantity of carbon monoxide produced. The nature of the changes which occur on the exposure of oxyhemoglobin to carbon monoxide is illustrated by the curves obtained in Fig. 4.

Whereas the test with hemoglobin was qualitative, an estimation of · the sensitivity of the test may be indicated. Considering the hemoglobin content of bovine blood to be approximately 15%, 0.15 g. of
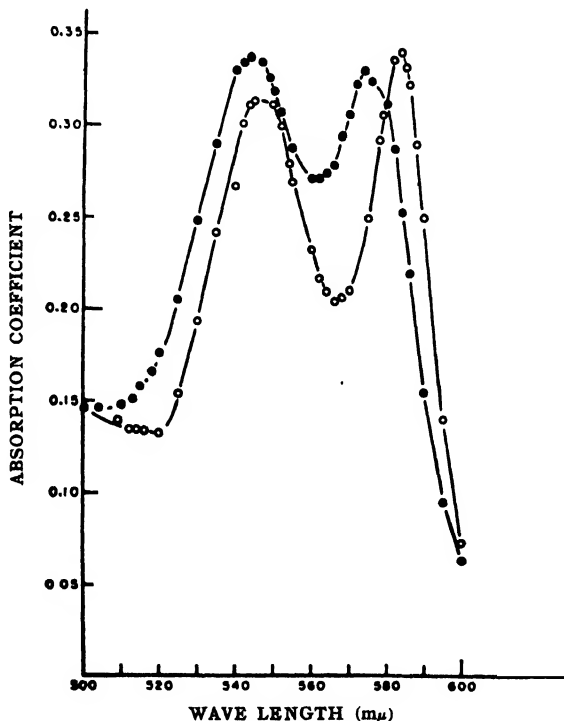
FIG. 4. ○ Absorption curve for oxyhemoblogin;
● absorption curve for carbon monoxide hemoglobin.

hemoglobin were exposed to the stream from the irradiation chamber. This corresponds to approximately 2×10^{-6} mols of hemoglobin. About 8×10^{-6} mols of carbon monoxide would then be required to completely convert the oxyhemobolbin to carbon monoxide hemoglobin. There is not the slightest suggestion of a maximum at 576 mμ in the lower curve of Fig. 3; thus, under the conditions employed by us, no detectable quantity of carbon monoxide was produced.

Determination of Hydrogen

In the experiments for the determination of hydrogen, the sodium hydroxide tower was replaced with a phosphorus pentoxide tube, and the chamber immediately before the second combustion chamber was replaced with a sulfuric acid tower. The irradiation of the cellulose was continued for 16 hours, while the nitrogen stream was passed through the apparatus. Since there was not a measurable change in the weight of the P_2O_5 tube in each of 5 determinations, we have concluded that, if hydrogen is produced on irradiation, it is produced in very small quantities.

Post-Irradiation Effect

In the experiments reported herewith it was demonstrated that carbon dioxide is liberated from the cellulose as a post-irradiation effect. In the experiments, the cellulose was exposed to ultraviolet irradiation in an atmosphere of nitrogen and, after a period of 16 hours, the lights were turned off. The entire apparatus was then washed with a stream of nitrogen for a period of 20 hours, after which air was passed through the apparatus for 8 hours. The quantity of carbon dioxide recovered during the 20 hour washing period was not large enought to be measured. However, after the 8 hour period an average (3 determinations) of 0.0108 millimols of carbon dioxide/g. of cellulose were recovered.

Production of Carbon Dioxide With Regenerated Cellulose

The evidence which we have obtained, and that which we have found in the literature, is not sufficient to formulate an hypothesis for the effect of the ultraviolet irradiation. We have no notion concerning the source of carbon dioxide from the cellulose irradiated in the absence of free oxygen. There is evidence that the changes which occur are changes in the cellulose, since we have observed that cotton cellulose which has been regenerated from cupriethylenediamine solutions under conditions which should eliminate cell constituents (the cotton fiber is an epithelial cell) such as may occur in the lumen of the cotton fiber, likewise produces carbon dioxide on irradiation with ultraviolet light. The quantities of carbon dioxide were comparable to those obtained with the dewaxed cotton fibers.

SUMMARY

1. Cotton cellulose in cupriethylenediamine solution shows a weak absorption in the region 370–400 mμ.

2. Irradiation of cotton cellulose in cupriethylenediamine solution causes a reduction in the viscosity, but it does not modify the absorption spectrum.

3. Carbon dioxide is produced in appreciable quantities on the ultraviolet irradiation of dewaxed cotton in an atmosphere of nitrogen.

4. Carbon dioxide was produced on ultraviolet irradiation of cellulose which was regenerated from cupriethylenediamine solutions of cotton cellulose.

5. There was no evidence for the production of hydrogen, of carbon monoxide or any other volatile carbon compound on the irradiation of cotton cellulose with ultraviolet light.

6. A post-irradiation production of carbon dioxide was obtained passing air over cellulose which had been irradiated in a nitrogen atmosphere.

Further Observations Concerning the Relationship of Temperature of Blanching to Ascorbic Acid Retention in Green Beans[1]

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INTRODUCTION

In a former study, relative to the effect of duration and temperature of blanching on the amount of vitamins retained by certain canned vegetables (1), it was observed that green beans blanched at 160°F. for 1, 3, or 5 mins. retained lower percentages of their original ascorbic acid than did similar beans blanched for the same periods at 180°F., at 200°F., or after steam blanching. Although repeated experimentation yielded data which substantiated the above observations, the explanation for this anomaly was not apparent. More recently, Wagner *et al.* (3) have reported somewhat similar observations. Consequently, additional blanching studies have been carried out with the view of throwing further light on this apparently unusual effect of blanching temperature on ascorbic acid retention.

EXPERIMENTAL

Experiment 1

A quantity of freshly harvested green beans (Kentucky Wonder, having an initial reduced ascorbic acid content of 18.07 mg./100 g.) were selected as the product for study. The whole beans were rinsed, drained, snipped, broken into quarter-lengths, and mixed so as to form a uniform lot. Weighed amounts of the broken beans were blanched in fresh portions of distilled water (one lb. of beans/gal. of water) for 3 mins. at 100, 120, 140, 150, 160, 170, 180, 190, and 200°F. The blanching was carried out by suspending the beans in loosely held cheesecloth, immersing the cloth and

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contents in the blanching water, agitating gently during the blanching period, and at the same time maintaining the water at a constant temperature. The beans were removed from the blanching water, drained for one min. and weighed. Samples of the blanched beans were taken immediately for reduced ascorbic acid determination, and the remaining beans were transferred to a glass dish and placed in a low temperature° freezer (−8°F.). When the temperature of the blanched beans reached the freezing point, other samples were taken for ascorbic acid determination. Ascorbic acid determinations were also made on aliquots of the blanching water. The effect of varying the temperature of the blanching water on ascorbic acid retention is shown in Fig. 1.
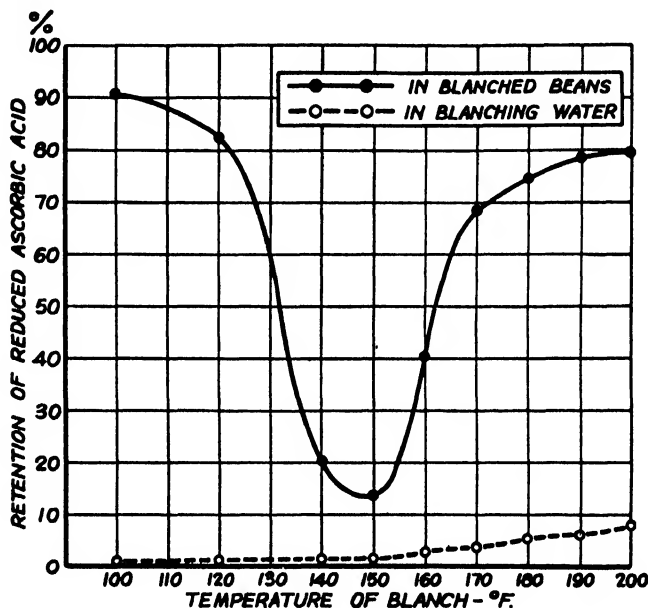
Fig. 1. The effect of the temperature of the blanching water on the amount of reduced ascorbic acid retained by green beans.

It will be noted that the amount of vitamin in the blanching waters increased consistently with increase in blanching temperature, but under no condition did it exceed 8.1% of the ascorbic acid originally present in the unblanched beans. The amount of reduced ascorbic acid accounted for in the blanched beans decreased as the blanching temperature increased from 100 toward 160°F., and then increased with increases in blanching temperature. For instance, 90.3% of the original reduced ascorbic acid was found in the beans blanched at 100°F. for 3 mins., 13.7% in those blanched at 150°F., and 79.8% in those blanched for a like period at 200°F. Tests for peroxidase activity

in the blanched beans revealed that the beans blanched at 170°F. and above showed no activity, those blanched at 160°F. showed slight enzyme activity, and those blanched at 150°F. and below showed vigorous peroxidase activity. While no explanation for this unusual loss in reduced ascorbic acid was apparent, the data did suggest that an enzyme system was involved. Therefore, further studies were conducted with the view of clarifying this point.
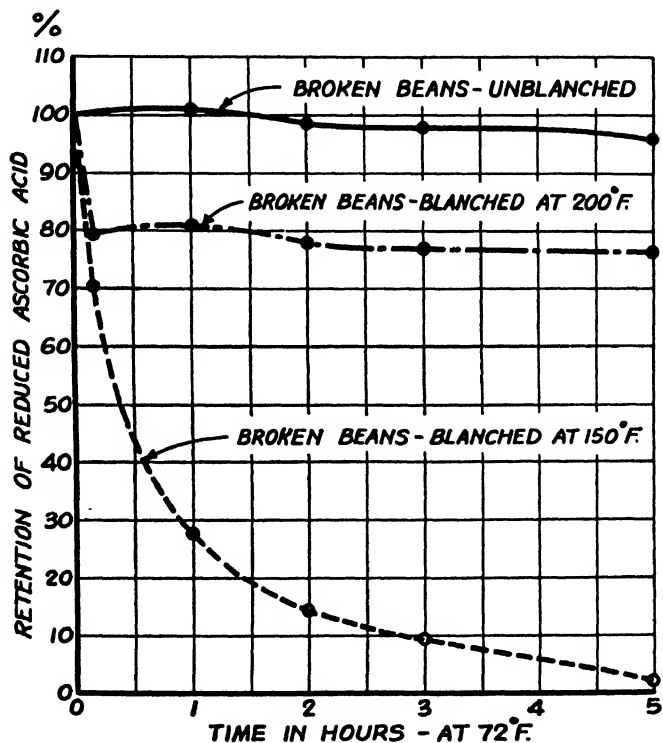
Fig. 2. The effect of holding unblanched and blanched green beans at room temperature on reduced ascorbic acid content.

Experiment 2

A second portion of freshly harvested pole beans (from the same vines as those previously used, but somewhat more mature and containing 20.97 mg. reduced ascorbic acid/g.) were prepared as previously described and divided into 3 equal portions. One portion was sampled immediately for the reduced ascorbic acid determination and the remainder of that portion allowed to stand at room temperature (72°F.) under

ordinary lighting conditions. Additional samples were taken for ascorbic acid determinations at the end of 1, 2, 3, and 5 hrs. A second portion of beans was blanched for 3 mins. at 200°F. (one gal. of distilled water/lb. of beans), drained, cooled rapidly to room temperature, and sampled for ascorbic acid analysis immediately and agair after standing for 1, 2, 3, and 5 hrs. The third portion of broken beans was similarl₃ treated except that the blanching was carried out at 150°F. The results of these test₁ are shown in Fig. 2.

It will be noted that the reduced ascorbic acid content of the broken, unblanched beans remained remarkably constant during the 5-hr. exposure at room temperature. In fact, during this period less than 5% of the original ascorbic acid was lost. When the beans were blanched for 3 mins. at 200°F., approximately 21% of the ascorbic acid disappeared during the blanching operation, but the remaining ascorbie acid was relatively stable during the next 5 hrs. However, when the blanching was carried out at 150°F., approximately 30% of the ascorbic acid was lost during the blanching operation and the loss of vitamin continued throughout the 5-hr. period. One hr. after the beginning of the blanching operation, the beans contained 28% of their original content of reduced ascorbic acid, and 4 hrs. later contained only 2.2% of that amount.

Thus, it became evident that the loss of ascorbic acid from the blanched beans was not due to exposure to air, but apparently was associated in some manner with an enzyme system which seemed to be activated by blanching at the lower temperatures (130–160°F.) for a specified time. The data show that the blanching of beans at 200°F. served to stabilize the remaining ascorbic acid present in the blanched product, and therefore this temperature must have been effective in altering the enzyme system. These findings suggested that, perhaps, a longer blanching period at 160°F. would result in a greater retention of the vitamin owing to a more thorough penetration of heat into the pieces of broken beans. Therefore, a repetition of the foregoing experiments, using more extended blanching periods (more than 3 mins.), seemed to be in order. However, it was believed that the desired information could also be obtained by carrying out the blanching under the conditions already outlined, but using batches of beans from a common source and breaking the beans constituting each batch into different lengths, so as to vary the amount of surface exposed to the blanching water.

Experiment 3

A third batch of beans (purchased on the local market, initial ascorbic acid content: reduced, 12.1 mg./100 g., total, 15.1 mg./100 g.) was used in these studies. The beans were rinsed, drained, snipped, and mixed. One portion of the snipped beans was analyzed, before blanching, for reduced ascorbic acid and for total ascorbic acid by the method of Roe *et al.* (2). Another portion was blanched in the usual manner (3 mins. at 150°F.), drained and the ascorbic acid content was determined immediately after blanching and again after 2 hrs. of frozen storage. Similar portions of the snipped beans were broken into ½, ¼, ⅛, and ₁₆ lengths, respectively, blanched and analyzed for ascorbic acid. The results of this study are presented in Table I.

TABLE I

The Effect of Size of Pieces on the Amount of Ascorbic Acid Retained by Blanched Green Beans

Sample number	Treatment	Ascorbic acid content immediately after blanching (mg./100 g.)		Ascorbic acid content after 2 hrs. frozen storage (mg./100 g.)		Percent of ascorbic acid retained before or immediately after blanching		Percent of ascorbic acid retained after blanching and 2 hrs. frozen stourge	
		Reduced	Total	Reduced	Total	In reduced form	In oxidized form	In reduced form	In oxidized form
1	Unbroken-unblanched	12.1	15.1	—	—	79.9	20.1	--	--
2	Unbroken-blanched	9.5	15.6	5.0	13.8	60.7	39.3	36.1	63.9
3	Broken-½-blanched	9.5	14.3	4.6	13.4	66.2	33.8	65.9	65.9
4	Broken-¼-blanched	9.5	14.4	4.4	13.3	65.8	34.2	33.1	66.9
5	Broken- ⅛-blanched	8.6	13.2	4.4	11.6	64.9	35.1	38.0	62.0
6	Broken- ₁₆-blanched	6.5	10.8	3.1	9.8	60.5	39.5	31.6	68.4

It will be noted that the degree of subdivision of the beans, preliminary to blanching, did not bear a linear relationship to the amount of ascorbic acid retained by the blanched beans. In fact, the data do not show that the anticipated increased rate of heat penetration into the smaller bean particles resulted in an increase in ascorbic acid retention. Actually, there was somewhat greater loss of ascorbic acid (both reduced and total) from the more highly subdivided beans. Part of this decrease in vitamin retention was accounted for in the blanching water. The data show that the retention of reduced ascorbic acid decreased more rapidly with the increase in the subdivision of the bean than did the decrease in total ascorbic acid. It was of interest to note that, under all conditions of blanching, about ⅔ of the total ascorbic acid retained immediately after blanching was in the reduced form, whereas after 2 hrs. of frozen storage only ⅓ of the retained ascorbic acid was found to be in the reduced form. While this loss in the reduced form of the

vitamin was considerably less than that observed for similarly blanched beans held at room temperature for 2 hrs., the data clearly indicate the ineffectiveness of low-temperature storage in overcoming the chain of reactions initiated during the blanching operation. Undoubtedly, a' longer period of frozen storage would have resulted in a further reduction in the amount of reduced ascorbic acid retained by the blanched beans and an increase in the amount of the vitamin unaccounted for by both methods of assay.

The data do not explain the mechanism by which the reduced ascorbic acid present in green beans is so rapidly converted to the oxidized form of the vitamin following low-temperature blanching. However, the data strongly suggest that the reaction is the direct result of an enzyme system set into motion by the low-temperature blanching of the plant cells, or that it is caused by an oxygenic substance formed by enzyme action. The persistence of the reaction during low-temperature storage would seem to support the latter view. Moreover, the data indicate to a certain extent the wide discrepancy that may be found in ascorbic acid values as determined by the 2 methods of assay. It it is assumed that the partially oxidized form of the vitamin is biologically active and that the assay method employed yielded results which are a true measure of the amount of this form of the vitamin present in the blanched beans, it follows that the ascorbic acid values obtained by the dye titration method may be of questionable significance as far as ascertaining the true nutritive value of blanched green beans is concerned. Perhaps there are other vegetables which react similarly to green beans when blanched at the lower temperatures. The data further emphasize the importance of high temperature blanching in promoting maximum retention of ascorbic acid.

SUMMARY

The results of this study show that green beans retained little of their ascorbic acid when blanched for 3 mins. at 140, 150, or 160°F., whereas, when blanched at 180, 190, or 200°F. for the same period, they retained a high percentage of this viatmin. The decrease in reduced ascorbic acid could not be accounted for in the blanching water. The data suggest that an enzyme system is involved. Additional data show the reduced ascorbic acid content of green beans to be less stable

during a 5-hr. storage period, at room temperature, when blanched at 150°F. than when blanched at 200°F., or when the beans were unblanched. While there was an appreciable loss in total ascorbic acid, a more significant change was the increase in the dehydro form of the vitamin at the expense of the reduced form.

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Crystalline Alcohol Dehydrogenase from Horse Liver

In 1938 Lutwak-Mann (1) reported the partial purification of horse liver alcohol dehydrogenase. The enzyme exhibited certain differences from that crystallized by Negelein and Wulff (2) from yeast, most outstanding being the much slower rate of alcohol oxidation. We wish to report here the crystallization of the horse liver enzyme and some preliminary observations on its activity.

Using a slight modification of Lutwak-Mann's early steps, the liver was extracted with 2 volumes of water, centrifuged, and heated at 52°C. for 15 mins. After centrifuging, the enzyme was precipitated by adding an equal volume of acetone. The precipitate was then dried.

The dried powder was dissolved in water, giving a solution which contained considerable quantities of hemoglobin and some catalase. This solution, prepared from 5 kg. of horse liver, contained 1.2 g. of enzyme, degree of purity 0.01 referred to pure enzyme taken as 1.

Fractionation with ammonium sulfate brought to pH 7.5 by addition of ammonia, yielded a fraction soluble at 0.5 and precipitated at 0.7 saturation which contained 980 mg. of enzyme, degree of purity 0.08. The fraction precipitated below 40% contained the catalase while that still in solution above 0.7 saturation included much of the hemoglobin.

The enzyme was now fractionated with ethyl alcohol at -10°C. in 0.01 M phosphate buffer at pH 6.8. The fraction collected between 30 and 50 vols.-% contained 814 mg., degree of purity 0.2. Electrophoresis of this fraction demonstrated the presence of 4 components. The enzyme was the most electronegative of these.

Fractionation with ammonium sulfate at pH 5.7 was now carried out on the alcohol precipitate. The protein collected between 60 and 80% saturation contained 560 mg. of enzyme, degree of purity 0.6. Electrophoresis of this fraction showed a basic protein containing the activity, and two other small components one of which was hemoglobin.

A second alcohol fractionation under the same conditions as above, gave, between 40 and 60%, a precipitate containing 500 mg. of enzyme,

degree of purity 0.8. This precipitate was dissolved in water and dialyzed. Ammonium sulfate was added to give 50% saturation and the solution was centrifuged. Further addition of small amounts of saturated ammonium sulfate (0.55) resulted in the appearance of crystals of enzyme containing 70% of the total activity of the solution.

FIG. 1. Crystalline alcohol dehydrogenase from horse liver ($\times$ 900).

Recrystallization yielded a precipitate containing all the enzyme activity of the solution. The crystals are colorless and show the usual absorption band at 280 mμ. No other absorption band was seen.

Preliminary activity tests confirm the results of Lutwak-Mann concerning the low activity of the enzyme. With an alcohol concentra-

tion of 10^{-1} M/l. 0.150 mg. DPN/ml., and assuming the molecular weight of the dehydrogenase is 70,000, 1 molecule of dehydrogenase converts 220 molecules of DPN/min. to reduced DPN.

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  Received March 31, 1948

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Book Reviews

Proteins and Amino Acids in Nutrition. Edited by MELVILLE SAHYUN, Detroit, Mich. Reinhold Publishing Corporation, New York, 1948. xvi + 566 pp. Price $7.50.

This is a useful book. It contains 15 chapters, each by a carefully selected authority on some phase of protein chemistry in relation to nutrition, together with an appendix which consists of two tables, one showing the proximate composition of American food materials, and a second giving the nutritive value of 100 g. of the edible portion of a large number of selected foods. The first of these is quoted from Chatfield and Adams's *U. S. Dept. Agr. Circular No. 549*, published in 1940, the second is from *U. S. Dept. Agr. Misc. Pub. No. 572*, published in 1945; together they occupy 86 pages of the volume and make readily available a vast amount of detailed information about food materials. There is an introduction by Professor H. B. Lewis of the University of Michigan who says, in part, "It is hoped that the varied discussions in this volume, presented by workers in their respective fields, may contribute in some part to the solution of some of these problems and, in turn, suggest others. The answers to many of the questions of the biological role of the proteins are not yet available but the discussion should stimulate further investigation."

The problems to which Professor Lewis refers include the biological value of proteins and its relationship to the amino acid composition, the role of the proteins of the plasma, the enzymes, hormones, antibodies and viruses, the protein requirement in the diet of man and the proper management of protein nutrition in disease. Each of these topics is authoritatively discussed in the various chapters. Space does not permit mention of each chapter, but the topics range from an historical introduction by Elliot F. Beach, and an exhaustive treatment of the biological utilization of proteins by H. H. Mitchell, through discussions of the economic aspects of food proteins by Lela E. Booher and thoroughly practical statements on protein nutrition in pediatrics and pregnancy by S. Z. Levine, and in surgical patents by Charles C. Lund and Stanley M. Levenson, to a highly technical discussion of virus proteins by Max A. Lauffer. One of the outstanding chapters is that by Abraham White on the relation of hormones to protein metabolism.

Only a few errors were noted, and none of these is serious; to mention one, on p. 135 there is a statement concerning the "twenty-odd plant proteins isolated by Osborne". This is a misapprehension which probably arose from counting the names given by Osborne in a list of well-characterized plant proteins. Actually, Osborne investigated no less than 32 different plant seeds, from each of which more than one, and frequently several, readily distinguishable protein preparations were isolated.

As a whole, the book is to be highly recommended. It presents an extremely valuable summary of the present position of knowledge of the nutritive effects of proteins, and it will doubtless find wide use in the teaching of the subject.

<div style="text-align: right">H. B. VICKERY, New Haven, Connecticut</div>

Heavy Metals As Active Groups Of Enzymes. By OTTO WARBURG. Berlin-Dahlem. Published by Dr. Werner Saenger, Berlin, Germany, 1946.

This is a book of 195 pages written in German. It contains such a wealth of material that a comprehensive review is difficult. The subject matter is divided into 21 chapters which summarize nearly all of the research of Warburg and his associates. The book is written in a clear and simple manner and the reviewer has been greatly impressed by Warburg's forceful and logical manner of expression.

As can be seen by the title, Warburg covers the role of iron and other heavy metals in catalyzing cellular oxidations. His fundamental theme is that certain living cells contain a water-insoluble oxygen-transferring enzyme which is oxidized by gaseous oxygen and which is reduced by metabolites in the cells. This oxygen-transferring enzyme is an iron compound which bears a close relationship to hemoglobin. In the ferric state it combines with hydrocyanic acid, thereby becoming inactive. In the ferrous state it unites with carbon monoxide to become inactive also. This carbon monoxide compound is dissociated by light. By plotting the absorption coefficient against the wave length employed, Warburg has obtained an absorption spectrum which bears a remarkable similarity to the spectrum obtained with carbon monoxide hemoglobin. One difference is that the epsilon peak is much higher for the oxygen-transferring enzyme.

Warburg covers much of the history of physiological oxidation and includes work by Davy, Claude Bernard, MacMunn, Wieland, Theorell, Keilin, Dixon and others. He believes the oxygen-transferring enzyme contains a special sort of hematin which he has not yet isolated but which he believes will be isolated eventually.

Some of the other subjects which are considered in the book are: cytochromes and their historical development, the effect of carbon monoxide upon fermentation and oxidation, the catalytic oxidation of cysteine, the calculation of photochemical dissociation constants, the chemical constitution of various hematins, etc.

It would appear to the unbiased observer that Warburg's discoveries are of great value and importance but that the last word about cellular oxidations has not yet been written.

In conclusion the reviewer wishes to recommend Warburg's "Heavy Metals As Active Groups of Enzymes" as a work of great interest and value to chemists and biologists and as a work that will be considered a classic notwithstanding how many years have elapsed since its publication.

JAMES B. SUMNER, Ithaca, New York

Modern Cereal Chemistry, 4th ed. By D. W. KENT-JONES and A. J. AMOS, analytical and consulting chemists. The Northern Publishing Co. Ltd., Liverpool, England, 1947. vii + 651 pp. Price 50s.

This volume comprises 18 chapters which cover the composition of wheat and other cereals and of milled products; their nutritive value; flour strength and dough testing; wheat conditioning; bleaching and flour improvers; chemistry of baking processes; microbiology of cereals; and various analytical methods, including vitamin assays. While the authors obviously could not treat all of these subjects exhaustively within the compass of this one volume, a vast amount of material has been included. In general, the material has been carefully chosen, although at times the authors have

departed from the "modern" phases to include early contributions that now have merely historical interest.

Chapter II, entitled "Principal Wheats of the World," actually covers more ground than the title implies, since it includes a discussion of wheat diseases, foreign seeds found in commercial wheats, insect pests, and fumigation practices. The latter subject is becoming increasingly prominent in the programs of the cereal technologist, flour miller, and milling machinery designer and manufacturer. Both miller and baker are now more alert than ever before to the considerations of plant sanitation, as it concerns insects and rodents, and in many establishments the supervision of sanitary practices is assigned to the chemist, with the counsel of entomologists.

The reviewer commends particularly the inclusion of Chapter XV on the microbiology of cereals. This is a phase of cereal technology that has been somewhat neglected until recently, and even now it merits more exhaustive study. It is a phase with which one of the authors has been concerned in his own researches, and doubtless later editions of this book will expand the area of treatment of this subject. Thus, it appears that the manuscript went to press before the results of extensive studies of the probable role of microorganisms in the storage of damp wheat, conducted at the Minnesota Agricultural Experiment Station, came to the attention of the authors.

Other chapters of the book are well done, and give evidence of the alertness of the authors to the growing volume of knowledge in the several sciences that are of concern to the cereal chemist, including physical chemistry and the colloidal state of matter, vitamins and nutrition, physics and mechanical dough testing, microbiology, and other ancillary divisions of science, and technology based thereon.

Such an encyclopedic treatment as is accorded this large subject in this fourth edition of the Kent-Jones and Amos book must be of great convenience to many who do not have opportunity to follow the extensive literature in this field. Little of significance that was published prior to 1947 has been overlooked, although, to be sure, not all of the vast literature could be reviewed in detail. Many leads to supplementary reading are afforded by the annotated listing of several hundred books and papers published in recent times, however. Thus the substance of the fields that are covered is in the book, and the interested reader is introduced to the sources for more detailed study.

C. H. BAILEY, St. Paul, Minnesota

Symposia of the Society for Experimental Biology, Vol. I, Nucleic Acid. J. F. DANIELLI and R. BROWN, editors. Cambridge University Press, Cambridge, 1947. vii + 290 pp. Price $7.50.

Seldom has a scientific gathering served such a useful purpose as the Symposium of the Society for Experimental Biology in Cambridge, 1946. The papers given there were compiled in the present volume. This work fills a serious need in that it is the first comprehensive review of the subject since the appearance of Levene and Bass' monograph in 1931. In recent years no individual author has ventured to cover the entire field, for studies on nucleic acids have become so numerous and have branched out so widely that probably no single author could cover all aspects adequately. The method of choice, therefore, is to have the outstanding men in the field report their specialty and their recent progress. This has been substantiated in the present book

in which nearly everything in the realm of nucleic acids has been covered by a leading authority. As a result, most of the reviews are superb and the book offers fascinating reading. The topics range from structural investigations by the organic and physical chemist to histological and embryological problems. Obviously, the contributors have attempted to explain their specialties in simple terms and plain language. They have fully succeeded in this aim. The scope of the book can be illustrated best by enumerating the articles.

The first group of papers deals with the chemical, enzymatic, and physicochemical investigations. It comprises the following contributions: The Structures of Nucleic Acids (J. M. Gulland); Structure and Synthesis of Nucleotides (B. Lythgoe and A. R. Todd); The Biological Synthesis of Purine Compounds (H. M. Kalckar); The Macromolecular Behavior of Nucleic Acids (W. T. Astbury).

The second section is devoted to reports of biological investigations on nucleic acids and nucleoproteins. It contains the following papers: The Distribution of Nucleic Acids in Tissues (J. N. Davidson); Bacterial Nucleic Acids and Nucleoproteins (M. Stacey); A Study of Techniques for the Cytochemical Demonstration of Nucleic Acids and Some Components of Proteins (J. F. Danielli); Ribonucleic Acid and the Vital Staining of Cytoplasmic Vacuoles in Animal Cells (P. Dustin, Jr.); The Relation between Nucleic Acid and Protein Synthesis (T. Caspersson); Protein and Nucleotide Metabolism in the Nerve Cell under Different Functional Conditions (H. Hydén); Nucleic Acid in Nerve-Cell Regeneration (D. Bodian); Tissue Changes in Mice Treated with Pentose Nucleotides (L. D. Parsons, J. M. Gulland and G. R. Barker); Histochemical Investigations on Nucleic Acids in Homologous Normal and Neoplastic Tissues (R. E. Stowell); Nucleic Acids in the Cell and the Embryo (J. Brachet); The Action of Enzymes on Chromosomes (D. G. Catcheside and B. Holmes); The Function of Desoxyribosenucleic acid in the Cell Nucleus (E. Stedman and E. Stedman); Nucleic Acid and the Chromosomes (C. D. Darlington); The Experimental Modification of Nucleic Acid Systems in the Cell (P. C. Koller).

There is no doubt that this book will become a classic of major importance in the field of biology and biochemistry.

F. SCHLENK, Ames, Iowa

Plants and Environment. By R. F. DAUBENMIRE. John Wiley & Sons, Inc., New York. xiii + 424 pp. with 87 figs. Price $4.50.

This is a text book of plant "autecology," which is defined by the author as that part of ecology dealing with the interrelationships between the individual plants and their immediate environments. He considers this to be basic to the more generalized ecology that deals with the structure, development, and distribution of plant communities. It is a little difficult to judge the audience for whom the book is intended. The general level would appear to be suited to junior or senior year students in botany though there are some portions that might be considered more advanced and some much less advanced. There are, for example, sections on the pH of solutions, and the determination of relative humidity that are very elementary. On the other hand certain portions of the section on light may be heavy going for students who have not had an advanced course in plant physiology. Over 600 literature citations (in English) are appended. These should be useful for reading assignments or to permit a student to expand his knowledge on particular subjects. The papers cited appear to have been carefully selected, and are a judicious mixture of the classical and the recent.

The book consists of 9 chapters, the first 5 of which deal with the major factors of soil, water, temperature, light, and atmosphere. Much of the material is not usually directly presented to students of ecology as a part of their ecological training. It is, in fact, an effective summarization of the material ordinarily included in courses in soils and plant physiology. The remaining 4 chapters on the biotic factor, the fire factor, the environmental complex, and on ecologic adaptation are more conventional and probably less inspiring.

Authors of text books are often charged with the perpetuation of obsolete theories; this charge cannot be levelled at Dr. Daubenmire, though he does repeat and define certain imposing terms that are rarely, if ever, employed. It appears that a commendable attempt has been made to include the newer viewpoints, and newer information. This book can be recommended to students and to others who need a generalized presentation of the information relating to the effects of the environment on plants.

A. G. NORMAN, Frederick, Maryland.

A New Notation and Enumeration System for Organic Compounds. G. MALCOLM DYSON. iv + 63 pages. Longmans, Green and Co., New York 3, N. Y., 1947. $1.75.

Progress in each branch of science depends partly on the effectiveness of the technical language invented for its purposes. That language may consist of words or of other symbols. Chemistry presents a special communication and recording problem because of the large number of known and potentially preparable compounds. The first effective nomenclature for chemical compounds was that introduced by Guyton de Morveau in 1782. Another landmark in chemical communication was the introduction by Berzelius, in 1813, of letter symbols and their use in the modern sense to denote proportion of elements in a compound. The development of structural organic chemistry has led to the need both for names and formulas for expressing structures and this need has been met reasonably well. However, for some purposes, as classification, indexing, and sorting by such modern devices as punched-card machines, structure designation by words or by the conventional linear or ring formulas is not wholly effective. The announcement of a new and promising method of expressing organic structures is accordingly a significant event, particularly in these days when there is such high interest in chemical record keeping and in the possibilities of the use of mechanical aids in obtaining information. The Dyson cipher has deservedly attracted much attention. A new language in a science can have much significance.

After a brief introductory discussion devoted principally to "some difficulties of existing nomenclature" this book is devoted to a description of the cipher with numerous examples.

The cipher undertakes to express structures linearly by the use of capital letters, Arabic numerals, and a few additional signs, as punctuation marks and brackets. It works. The author has proved this by ciphering a great many compounds, e.g., all in several volumes of Beilstein.

Ciphering by the Dyson system is not difficult to learn, though handling some of the more complex compounds cannot be called easy. The short linear ciphered "names" are well adapted for indexing. For classification without mechanical aid there are limitations inherent in the method of formation—as in other systems.

It is perhaps too early to make predictions as to the usefulness of this notational and enumerative system. It will hardly replace the conventional names or structural

formulas for everyday use, but it is certainly worthy of the further experimentation which is going on, both for its improvement and to determine its adaptability for special purposes. It will have "Sunday" use surely, and probably a good deal of everyday value.

E. J. Crane, Columbus, Ohio.

Ernährungsprobleme in Mangelzeiten. Die schweizerische Kriegsernährung 1939–1946. By Alfred Fleisch, "Ordinarius" for Physiology, Director of the Physiological Institute at the University of Lausanne, President of the Federated Wartime Nutrition Commission. Schwabe, Basel, 1947. 518 pp, 155 tables, 21 illus. Price 32 Sw. frs.

While the nutrients required for optimum nutrition are well known, the minimum amounts necessary to maintain health and capacity for work have not been determined. Data on this point could be obtained by an experiment in which at least 1000 persons are placed on a controlled study for one to three years. At the beginning, a liberal diet, rich in animal protein and fats, would be fed. As the experimental period advanced the diet would be gradually changed to a starvation diet consisting mainly of vegetable protein, dark bread, vegetables, and fruits. The subjects would be closely observed to determine the time when unmistakable signs of malnutrition became evident as a result of the deterioration of the food supply. Changes in body weight, hemoglobin, resistance to disease, mortality rate, tuberculosis incidence, fatigability, and feeling of well-being would be among the useful criteria used in such a study. It so happens that this type of controlled experiment was forced upon Switzerland and her 4,300,000 population during World War II, and Alfred Fleisch reports the results in this book.

Before the war Switzerland imported a major portion of its wheat and sugar, and approximately half of its calories from abroad. Although a neutral nation, Switzerland had many problems with her food supply. Tea and coffee were the first to be rationed. As the war continued, rationing was extended to more and more food items. In July, 1941, fats, then cheese, eggs, milk, and meat, were added. By the end of the war the extraction of bread had been raised to 90%; every food, except potatoes, was on the ration list. Coupons were given up when meals were eaten in restaurants. The rationing system was especially effective because there was no serious black market.

The country passed through a dark period late in the spring of 1945 when the *per capita* supply of calories fell to near 1800 daily; the intake of fat and protein fell to 40 g. and 54 g., respectively. Special supplements were given to workers in various categories, and allocations of certain foods to infants, children, invalids, and diabetics, were made. The sick tolerated the 90% extraction bread very well. Though the Swiss dietary was poorer than the above-minimum diets of the Anglo-Saxon countries, it was better than the below-minimum diets of the occupied countries. Thus, observations on the Swiss people during 1939 to 1946 are of world importance.

The following are the chapter titles: I. The problems of the Federated Wartime Nutrition Commission in controlling the federal wartime consumers offices; II. Dietary plan for wartime; III. Farm production realized during the war; IV. Management of important foods; V. Rationing methods; VI. Physiological and nutritional bases for rationing; VII. The rations during 1943–1946; VIII. Comparative considerations on nutrition during war; IX. Provisions of vitamins and minerals; X. Flavor and satiety values of war rations; XI. The deficiency diseases; XII. Social

problems; XIII Teaching the people about nutrition; XIV. Basis for nutrition and health; XV. Post-war nutrition.

This discussion of the food problems of a country during a trying period is of historic value, not only to the Swiss, but to those interested in rationing and in the nutrition of masses of people. It is perhaps the most complete account of the techniques and effects of food rationing that has been published. The changes in hemoglobin and in weight observed in the Swiss population during the war period seemed to be related to variations in the rations. It is important that these observations be confirmed and the relationship be established on another population group.

The author was president of the Federated Wartime Nutrition Commission in Switzerland during the war and is peculiarly qualified to discuss the many food problems during periods of stress, the importance of efficient land cultivation and crop selection, the functions of the food industries, the impact of trade politics and social problems, and especially the relationship between disease and nutrition.

<div style="text-align:right">ROBERT S. HARRIS, Cambridge, Mass.</div>

Tabulae Biologicae, Vol. XXII (Oculus), Part I. By Dr. W. JUNK. Amsterdam, 1947. pp. viii + 408.

Tabulae Biologicae has undertaken to publish a monumental compilation of data on the eye and vision, of which the first of 4 volumes has now appeared. Those familiar with other volumes of the *Tabulae* will recognize that what is attempted here is the collection of reported numerical and descriptive information, with little attempt at critical evaluation or discussion. The result takes the form in most articles of long tables of numbers and bibliographic references. Depending upon the discrimination of the author, these can be valuable and meaningful, or they can be just so much busywork. The present volume contains examples of both types of contribution.

This volume contains chapters on the anatomy of invertebrate eyes (H. Kahmann); metric and descriptive data on the eyes of living and prehistoric men, and other primates and mammals, primarily of anthropological interest (S. Oppenheim); the embryological development of the human eye (C. Dejean and F. Granel); the anatomy of the vertebrate eye (K. Steindorff); the vertebrate pupil (J. Nordmann); and intraocular pressures and blood pressures (K. W. Ascher).

With the exception of the final chapters by Ascher, which go into the physiology and pharmacology of intraocular and blood pressures, there is little in this volume of physiological or biochemical interest. Such matters have been held for the remaining three volumes, now in preparation and of more direct concern to the readers of this journal.

<div style="text-align:right">GEORGE WALD, Cambridge, Mass.</div>

Chromatographie et Mésomérie. Adsorption et Résonance. By PAUL MEUNIER, "Maître de Conférences" in Biochemistry at the University of Lyon, France, and (Miss) ANDRÉE VINET, "Chef de Travaux" at the School of Agriculture in Grignon, France. Masson and Co., Paris, 1947. 126 pp. Price, 280 French fr.

The first two chapters of this book serve as a brief introduction to the electronic theory of valence and resonance, which is used in the third chapter to gain a qualitative understanding of the absorption spectrum of organic molecules. In the fourth chapter the various forces responsible for adsorption on solids are discussed and con-

nected with electron formulations. The authors differentiate between the adsorption on the surface of ionic crystals like alumina or calcium carbonate, which is presumably due to electrostatic effects, and adsorption on acid earths, which is explained by formation of a covalent bond between the adsorbed molecule and the adsorbent. The latter case is the more interesting one because of the resulting changes in the adsorbed molecule, which in the case of compounds containing oxygen may lead to their dissociation into two parts and, on elution, to the formation of new compounds. The authors refrain from any detailed discussion of the sequence of various compounds on a Tswett column and mention only briefly the recent mathematical theories of chromatography. The distinction of two kinds of adsorption is along lines similar to that in the work of E. Weitz and collaborators [1] and especially recalls a discussion remark of G. Schwarzenbach.[2] Perhaps Weitz's ideas should have been considered more in detail in the book under review; however, only a brief reference is given on p. 92.

In Chapter 5 these notions are applied to special cases in the field of the carotenoids. The Carr-Price color reaction is interpreted, and the changes that vitamin A suffers when adsorbed on acid earths or by interaction with antimony trichloride are explained in terms of structural changes. A discussion of some stereochemical aspects of carotenoids closes the chapter.

The last chapter attempts an explanation of various color reactions and rearrangements of steroids, especially of some derivatives of ergosterol and 7-dehydrocholesterol. The book is concluded with an interesting hypothesis about the mechanism of Liebermann's reaction and the appearance of antirachitic activity on prolonged interaction of some steroids and Liebermann's reagent. These last two chapters contain a number of conjectures which will have to be tested experimentally before being generally accepted, but the value of the ideas proposed is precisely the formulation of such working hypotheses.

While Meunier anf Vinet's book doubtlessly contains a number of interesting and stimulating ideas, it seems to be frequently inaccurate in the treatment of details. The introductory chapters are in the main satisfactory, although the section on the alternation of the inductive effect (p. 13 ff.) is somewhat puzzling, all the more so since the examples cited could be easily explained without the assumption of such an alternation. The section on hyperconjugation is not very clear, but the discussion of resonance is satisfactory, with the following exceptions. It is improbable that the mesomerism cited on p. 25 plays any role in tautomerism. The reviewer believes that structures of the type $-\overline{CH-CH=CH-CH}-$ are of some importance to the ground state of a conjugated double bond system, and not ionic structures of the type $-\overset{\ominus}{CH}-CH=CH-\overset{\oplus}{CH}-$ (pp. 28, 45) which contribute to the excited states responsible for the spectrum; this is, indeed, pointed out in a footnote on p. 46. The statement (p. 44) that the smaller the difference between the limiting structures taking part in a resonance, the smaller the difference between the energies of ground and excited states, is misleading. The reason for a strong light absorption within or near the visible

[1] Z. Elektrochemie **47**, 65 (1941).
[2] Ibid., p. 72.

range of the spectrum is usually that resonance stabilizes the excited state (with large contributions from excited limiting structures) more than the ground state (representing mainly unexcited structures). It is the delicate balance between these *two* resonance stabilizations which determines, *e.g.*, the exact position of the absorption band in azulene (p. 45).

Again in Chapter 4 there are a number of statements at variance with our present picture of the structure of matter. Van der Waals forces and hydrogen bonds, besides the electrostatic and dipolar forces mentioned, are very much responsible for holding together the molecular units in the crystals of glycine, sucrose, cellulose, *etc.* The hydration of ions in solution is evidently due to electrostatic forces and not to sharing of electron pairs. The hydrogen bond does not involve resonance of the type cited but is the result of electrostatic forces. Calcium carbonate, calcium sulfate, and alumina crystallize with ionic lattices which do not contain the single molecules indicated in the formulas given on p. 57.

In the last two chapters of the book one sometimes wonders whether there might not be alternative formulations as likely as the ones proposed. One puzzling effect is, *e.g.*, the blue coloration of vitamin A interacting with $SbCl_5$ or $AsCl_5$ (footnote, p. 72) which appears analogous to the Carr-Price reaction involving $SbCl_3$. The explanation of the latter effect postulates that $SbCl_3$ acts as an electron acceptor which, however, could hardly apply to $SbCl_5$ or $AsCl_5$.

Although there is reason for some formal criticisms, only few misprints were noticed, and on the whole the book is stimulating. The well placed summaries are excellent, and each chapter contains an up-to-date bibliography. Misconceptions, the more important of which have been mentioned above, might be due to the lack of contact of the authors with some modern developments during the war. It is, indeed, surprising how many current ideas have been coordinated in this brief monograph which we hope will soon appear in a new and improved edition.

JÜRG WASER, Pasadena, Calif.

Glandes Endocrines et Vitamines. By Faculté de Médecine de Genève. Les Presses Academiques, Genève, 1943. 726 pp.

"Glandes Endocrines et Vitamins", Cours de, Perfectionnement organise par la faculté de Médecine de Genève, is a resume of 39 lectures delivered on the above subjects for a post-graduate course at the University of Geneva.

The lectures are very interesting but this book does not replace a text book on endocrinology or vitamins because it is incomplete in its detail. In addition it is not brought up to date since the lectures were delivered in 1942. Quite a few important observations have been added to our knowledge in this field since that time. The book is written in French and is illustrated with pictures.

S. J. THANNHAUSER, Boston, Mass.

The Chemistry of the Carbon Compounds. Volume. IV. By VICTOR VON RICHTER and RICHARD ANSCHÜTZ. The Heterocyclic Compounds, newly translated by M. F. Darken, and Organic Free Radicals, newly translated by A. J. Mee. 3rd English edit. based on the 12th German edit. Elsevier Publishing Company, Inc., New York, 1947. xv + 498 pp. 14 × 21.5 cm. Price $12.00.

The series known as the Richter Organic Chemistry texts needs no introduction to older organic chemists who are familiar with one or more of the 12 German editions

and with the one or more of the 3 English translations which were generally revised to bring them up to date. The present edition is a literal translation of Vols. III and II, pt. 2, which include heterocyclic compounds and free radicals, respectively, of the 12th German edition published in 1931 and 1935. The only departure from the German texts in this volume is that the references are to the original papers rather than to the *Chem. Centr.*, and that the names of the authors are included. Especially convenient for American chemists is the system of nomenclature, which is that followed in *Chem. Abstracts* and which is briefly reviewed.

The section on heterocyclic compounds deals extensively with rings containing from 3 to 8 or more atoms and with from 1 to 4 hetero atoms in the ring, as well as with fused systems. There is also a section on the alkaloids which contain heterocyclic nuclei.

The section on organic free radicals contains a lengthy account of trivalent carbon compounds as well as a reference to similar compounds prepared from silicon, tin, and lead. There is also a comprehensive account of free radicals containing tetravalent or divalent nitrogen as well as a number of diradicals. Finally, radicals of monovalent oxygen, tri- and monovalent sulphur, tetravalent chromium, and boron and aluminum are discussed.

Throughout the book a historical background is given for most classes of compounds, and the preparative procedures are adumbrated extensively. Physical properties are given for most substances.

The reviewer does not consider this volume suitable as a general text-book in organic chemistry. Its usefulness to advanced students of the subject, however, is quite apparent, particularly if teachers will prescribe the reading of certain chapters along with their lectures. The book bridges the gap between the text-books and lengthy review articles. It also serves as a sort of Beilstein and Richter's Lexicon where these are not accessible.

The book is remarkably free of misprints and has suffered nothing in the translation, while the numerous references to compounds of pharmaceutical and commercial interest will win it many readers.

RICHARD H. MANSKE, Guelph, Ontario, Canada

Organic Chemistry. By PAUL KARRER, Professor at the University of Zürich, Zürich, Switzerland. Third English ed., translated from the "latest" German edition by A. J. MEE, Glasgow Academy. Elsevier Publishing Co., Inc., New York, N. Y., 1947. xx + 957 pp. Price $8.50.

The third English edition of this well-known, comprehensive organic chemistry text follows very closely the lines of the second English edition, published in 1946· Professor Karrer states that every chapter has been the subject of careful scrutiny and revision, and that, where necessary, passages have been rewritten to bring them into line with the results of recent investigations, including the literature to about the end of 1945.

The strength of this text, as in previous editions, lies in its broad coverage of the facts of organic chemistry. Sections dealing with the structure and synthesis of natural products are especially strong, and the book will have a particular appeal to students interested in medical or biochemical applications of organic chemistry. Theories concerning relative reactivity and reaction mechanisms receive very little

attention, while structural organic chemistry, including stereochemistry, is treated concisely and skilfully. Probably no other book on organic chemistry which could be recommended as an elementary organic text covers such a wide range of subjects. It has been necessary to sacrifice completeness in detail to some extent in order to achieve this scope in a single volume. Sections of the book which will be useful as a text or reference for advanced courses include: terpenes, sterols, vitamins, heterocyclic compounds, and alkaloids. Unusual features include a section describing organic compounds containing heavy hydrogen and heavy oxygen, and a number of tables concerning such diverse subjects as compounds present in coal tar and important dates in the history of organic chemistry.

Professor Karrer's book has few close competitors in the field of comprehensive single volume textbooks of organic chemistry, and deserves the wide use which it undoubtedly will receive. It is of interest that the third English edition was printed in the Netherlands, and that the work of the printer was extraordinarily well done.

ARTHUR C. COPE, Cambridge, Massachusetts

Actualités scientifiques et industrielles. 981. IV. Les cancers produits par les rayonnements electromagnétiques. By ANTOINE LACASSAGNE Hermann et Cie., Paris, 1945.

This article on the production of neoplasms by radiation, reviews the early work on the production of tumors by radiations emitted by the naturally occurring radioactive elements when given externally by means of seeds or capsules, or after intravenous or subcutaneous injection. It also reviews the literature on the production of tumors in man whether as a result of the accidental ingestion of radioactive materials, such as occurred in the case of the radium dial painters, or after the therapeutic use of natural radioactivity which was in vogue at one period during the present century. He also discusses studies of lung cancer in the Joachimstal and Schneeburg miners.

The review does not include any reference to the extensive work carried out during the past five years on the carcinogenic effects of X-rays, gamma rays, neutron rays, and the various radiations from fission products, and the artificially produced radioactive materials.

JOHN H. LAWRENCE, Berkeley, California.

Chemistry of Vitamins and Hormones. By S. RANGASWAMI AND T. R. SESHADRI, Andhra University, Waltair, South India. 1946. 329 pp.

This relatively small book will doubtless be found of value, particularly to those who do not have access to more extensive literature and who wish to consult broad survey material.

The emphasis is, as the name suggests, on the chemistry of the physiological substances in question, with relatively little discussion of physiological effects and functioning. Assay methods for numerous vitamins and hormones are, however, outlined. The literature available to the authors evidently did not justify mention of microbiological methods except in a very few cases.

More attention is paid, quite naturally, to those vitamins and hormones which have been studied extensively from the chemical standpoint. Synthetic methods, where available, have been briefly discussed as has the elucidation of structure in several cases. Hormones of complex and unknown structure, though recognized as important,

are passed over very lightly. For example, the entire discussion of pituitary hormones occupies less than two pages.

Since the vitamin field is an especially active one, it is not surprising that the book should be out of date at the present time in a number of particulars. There is one point on which the authors may be criticized, even taking into account the date of writing and the difficulty of obtaining up-to-date literature. This has to do with vitamin A. Since other writers in the vitamin field have been careless on the same point, not too much blame should be shouldered by our Indian colleagues. On p. 20 they say, "Vitamin A seems to exhibit complete specificity of action," and they fail to differentiate and make clear the distinction between the two ways in which the term *vitamin A* is sometimes used, *viz.*, as the name for a specific substance, carotenol, and as a name for a biological activity possessed by carotenes, carotenol, homocarotenol, esters of carotenol and homocarotenol, *etc.*

Considering the date of writing (May, 1946) and allowing for some lag due to the lack of availability of literature, the contents of this book constitute a dependable and valuable summary.

<div style="text-align: right">ROGER J. WILLIAMS, Austin, Texas</div>

Ammonia Formation from Cystine Peptides and Dehydropeptides in Rat Liver Digests

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INTRODUCTION

L-Cystine (1), dichloroacetyl-L-cystine (2), and diglycyl-L-cystine (2) are enzymically degraded in rat liver digests with the production of hydrogen sulfide and nearly equivalent amounts of ammonia and pyruvic acid. All 3 substrates are degraded under comparable conditions at rates of the same order of magnitude (2). The metabolism of dichloroacetylcystine is of particular interest for, with the exception of certain members of one class of compounds, it is the only peptide lacking an α-amino group thus far observed which yields ammonia readily when digested with rat liver homogenates. The exception consists of the dehydropeptides, acetyldehydroalanine, chloroacetyldehydroalanine, and DL-chloropropionyldehydroalanine, which are enzymically hydrolyzed with the production of nearly equivalent amounts of ammonia and pyruvic acid (3). Of these 3 dehydropeptides, chloroacetyldehydroalanine is hydrolyzed most rapidly.

It was considered of interest to investigate the ammonia production in digests of other acylated cystine derivatives, and diacetyl-L-cystine and di-DL-chloropropionyl-L-cystine were prepared for this purpose. The amination product of the latter compound, namely, DL-alanyl-L-cystine was also prepared, while for further purposes of comparison, the corresponding dehydropeptides and saturated peptides of alanine and of serine were investigated under conditions similar to those for the cystine peptides.

EXPERIMENTAL PROCEDURE

Diacetyl-L-cystine

Eighteen g. of L-cysteine hydrochloride were dissolved in 200 cc. of 2 N KOH, and treated at 15°C. with small portions of a total of 10 g. of acetic anhydride. At the

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end of the reaction, the mixture was treated with 5 N HCl to a congo blue reaction, and evaporated *in vacuo* to dryness. The residue was extracted several times with warm acetone, and the combined extracts evaporated *in vacuo* to a thick syrup. The residue was taken up in 100 cc. of water, treated with an excess of sodium bicarbonate, a few drops of ferric chloride solution were added, and the solution aerated for 24 hrs. The solution was filtered, acidified to congo blue reaction with 5 N HCl, and evaporated *in vacuo* to dryness. The residue was extracted several times with warm acetone, and the combined extracts evaporated *in vacuo* to a thick syrup. Like Hollander and du Vigneaud (4), we noted that the completely dry material was now insoluble in absolute acetone. The residual syrup was dissolved in absolute alcohol and dry ether carefully added. The product appeared as a white, semi-crystalline precipitate, which was redissolved in alcohol and reprecipitated with ether in the form of tiny needles. The product was filtered, washed with ethyl acetate and with dry ether, and dried *in vacuo*. The yield was 8 g., or 30% of the theory. M.p. 120°C. with decomposition.

($C_{10}H_{16}O_6N_2S_2$) Calculated: N 8.6, S 19.8; found: N 7.9, S 19.4.

Two hundred mg. of the compound boiled with 10 cc. 2 N HCl for 2 hrs. under the reflux yielded 105 mg. of cystine with $[\alpha]_D^{20} = -202°$. Little or no racemization of the cystine had occurred as a result of the acetylation.

Dichloroacetyl-L-cystine (5), diglycyl-L-cystine (6), di-DL-bromopropionyl-L-cystine (5), and di-DL-alanyl-L-cystine (5) were prepared as described. A portion of each preparation was subjected to HCl hydrolysis, and each yielded cystine with $[\alpha]_D^{20}$ varying from $-202°$ to $-212°$ in 1 N HCl.

Di-DL-chloropropionyl-L-cystine was prepared as follows. 100 g. of DL-chloropropionic acid was refluxed with 200 g. of thionyl chloride for 3 hr. The acid chloride was obtained on fractional distillation (40–50° at 25 mm.), and was coupled with L-cystine (cf. 5). The ether extract yielded on evaporation a colorless oil. The latter was taken up in a little dry ether and treated with dry petroleum ether. Sheaves of long needles appeared immediately. M.p. 187°. ($C_{12}H_{18}O_6N_2S_2Cl_2$) Calculated: C 34.2, H 4.2, N 6.6, S 15.2, Cl 16.9; found: C 34.2, H 4.3, N 6.3, S 15.0, Cl 16.2. HCl hydrolysis yielded L-cystine in hexagonal plates with $[\alpha]_D^{20} = -205°$.

The preparation of acetyl-DL-alanine (7), chloroacetyl-DL-alanine, (8), glycyl-DL-alanine (8), chloroacetyl-DL-serine (9), glycyl-DL-serine (9), acetyldehydroalanine (3), chloroacetyldehydroalanine (3), DL-chloropropionyldehydroalanine (3), glycyldehydroalanine (3), and DL-alanyldehydroalanine (3) have been described.

The enzyme studies were performed by mixing 2 cc. of a fresh rat liver homogenate prepared in distilled water with 1 cc. of 0.2 M phosphate buffer at pH 6.8, plus 1 cc. of either 0.025 M neutralized substrate solution (based on one S atom and one optical form), or distilled water. The initial and final pH was 6.8. That concentration of homogenate was chosen which would yield a nearly linear relation between ammonia production and time over a period of 2 hrs. incubation at 37°C. Ammonia was determined by aeration into sulfuric acid traps followed by Nesslerization. Aerobic conditions prevailed. The data given in Table I are corrected for the tissue blanks, and are given in terms of $\mu M \times 100$ ammonia N evolved/hr./mg. total N in the 2 cc. of homogenate. Each determination was made in duplicate. Each substrate was in-

vestigated 3–5 times with different tissue preparations, and the results varied by approximately ±15%. The data in Table I represent the means.

TABLE I

Enzymic Production of Ammonia from Various Substrates in Rat Liver Digests

Substrate	$\mu M \times 100$ ammonia N evolved/hr./mg. total N in homogenate
L-Cystine	22
DL-Alanine	1
DL-Serine	0
Diacetyl-L-cystine	7
Acetyl-DL-alanine	0
Acetyldehydroalanine	24
Dichloroacetyl-L-cystine	18
Chloroacetyl-DL-alanine	0
Chloroacetyl-DL-serine	0
Chloroacetyldehydroalanine	180[a]
Diglycyl-L-cystine	21
Glycyl-DL-alanine	1
Glycyl-DL-serine	0
Glycyldehydroalanine	350[a]
Di-DL-chloropropionyl-L-cystine	0[b]
DL-Chloropropionyldehydroalanine	35
Di-DL-analyl-L-cystine	23
DL-Alanyldehydroalanine	340

[a] Practically identical results were obtained when 1 μM of sodium sulfide was added initially to the digest.

[b] No evidence of hydrogen sulfide. Identical results with di-DL-bromopropionyl-L-cystine.

Discussion

Cystine, dichloroacetylcystine, diglycylcystine, and dialanylcystine yield ammonia in rat liver digests at rates of the same order of mganitude (Table I). Diacetylcystine yields ammonia at a definitely lower rate, whereas dichloropropionylcystine yields no observable ammonia at all under the conditions employed. When the last-mentioned peptide is aminated, forming dialanylcystine, its susceptibility to enzymic attack is greatly increased. Glycyldehydroalanine and alanyldehydroalanine are rapidly hydrolyzed at close to the same rate, whereas chloroacetyldehydroalanine is hydrolyzed somewhat more slowly, while acetyldehydroalanine and chloropropionyldehydroalanine are split at a still lower rate (Table I) (*cf.* 3).

The metabolism of the cystine peptides may follow either or both of two paths. Each path may be considered a two-step reaction, whereby ammonia and pyruvic acid are finally formed. Path I may consist (a) of a hydrolysis at the peptide bond by some peptidase leading to the production of the acyl acid and cystine, followed (b) by the desulfuration of the cystine by cystine desulfurase, leading to the production of hydrogen sulfide, ammonia and pyruvic acid. Path II may consist (a) of a desulfuration of the cystine while it is still in peptide combination by exocystine desulfurase, leading to the forfnation of the corresponding dehydropeptide plus hydrogen sulfide, followed (b) by the action of dehydropeptidase upon the dehydropeptide with the production of equivalent amounts of ammonia and pyruvic acid (2). According to Path II, the desulfuration of diacetylcystine would lead to the formation of acetyldehydroalanine, the desulfuration of dichloroacetyl-cystine would lead to the formation of chloroacetyldehydroalanine, *etc.* Since ammonia is the product which has been measured (Table I), it is the overall rate of the 2 possible reactions which is given in each case.

There exists no unequivocal evidence whereby one may choose between Paths I and II, but while there may be a possibility that both paths may account for the metabolism of diglycylcystine and dialanyl-cystine, it is less likely that Path I is concerned with the metabolism of diacetylcystine and dichloroacetylcystine. Since chloroacetyldehydroalanine is hydrolyzed at a rate considerably greater than that of either acetyldehydroalanine or chloropropionyldehydroalanine (3) (Table I), it would be expected, if there were some relation between the metabolism of the cystine peptides and their corresponding dehydropeptides, that dichloroacetylcystine would be metabolized at a rate considerably greater than that of either diacetylcystine or dibromopropionylcystine. This is indeed the case (Table I). While it cannot be said that these observations necessarily prove the relation suggested, they are of interest in this connection. Like dibromopropionylcystine, which forms on amination the highly susceptible dialanylcystine, chloropropionyldehydroalanine on amination forms the highly susceptible alanyldehydroalanine. In the latter case, the difference in susceptibility may be due to the fact that two different dehydropeptidases are involved (3). The possibility that more than one desulfurase may exist is not excluded.

Since hydrogen sulfide has been noted to be toxic to purified prep-

arations of dehydropeptidase (10), its action in digests of glycylde-hydroalanine and chloroacetyldehydroalanine with liver homogenates was studied (Table I). No effect was observed on the hydrolysis of these substrates with concentrations of sulfide in excess of those which might be derived from the desulfuration of the susceptible cystine peptides.

The final proof of the validity of the suggested Path II will depend upon the preparation of an exocystine desulfurase free of dehydropeptidase activity. Since the latter is so highly active, some difficulty in effecting such a separation may be expected to be encountered.

In contrast with the relative ease with which cystine and certain of its peptides yield ammonia in rat liver digests is the nearly complete resistance of alanine, serine, and their peptides (Table I).

SUMMARY

The enzymic production of ammonia has been studied in rat liver digests of diacetyl-L-cystine, dichloroacetyl-L-cystine, diglycyl-L-cystine, di-DL-chloropropionyl-L-cystine, and di-DL-alanyl-L-cystine. The rate of this production has been compared under similar conditions with analogous peptides of DL-alanine, DL-serine, and dehydroalanine.

Dichloroacetyl-L-cystine, diglycyl-L-cystine, and di-DL-alanyl-L-cystine yield ammonia at nearly the same rate. Diacetyl-L-cystine is less susceptible, and di-DL-chloropropionyl-L-cystine yields no ammonia. Chloroacetyldehydroalanine is hydrolyzed at a rate considerably greater than that of acetyldehydroalanine and of DL-chloropropionyldehydroalanine, although these rates are much below those for glycyldehydroalanine and for DL-alanyldehydroalanine. No effect of hydrogen sulfide on the rate of hydrolysis of dehydropeptides in rat liver homogenates has been observed. The possible paths of metabolism for the cystine peptides is discussed.

DL-Alanine, DL-serine, and their peptides yield little or no ammonia under conditions whereby peptides of cystine and of dehydroalanine are readily metabolized.

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Acetylated Dehydroamino Acids

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INTRODUCTION

The greater part of the work on the metabolism of dehydropeptides
has been concerned with peptides of dehydroalanine and of dehydro-
phenylalanine. Relatively little has been done with peptides of other
α,β-dehydroamino acids. Fruton, Simmonds and Smith (1) studied the
metabolism of acetyldehydrotyrosine, acetyldehydrophenylalanine,
acetyldehydroleucine, and acetyldehydroalanine in growing cultures of
Escherichia coli, and noted that only acetyldehydrotyrosine and ace-
tyldehydroalanine were metabolized.

Acetyldehydroalanine is metabolized in extracts of rat kidney and
liver, whereas acetyldehydrophenylalanine is not affected (2). With
the intention of extending these studies with animal tissues to other
acetylated dehydroamino acids, a series of such compounds was pre-
pared and investigated with digests of rat kidney and liver.

EXPERIMENTAL

Acetyldehydroalanine (3), acetyldehydrophenylalanine (4), acetyldehydrotyrosine
(4), and acetyldehydroleucine (5) were prepared as described. The preparation of
acetyldehydroleucine by Doherty, Tietzman and Bergmann (5) involves the con-
version of chloroacetylleucine to the corresponding azlactone by warming with acetic
anhydride, followed by hydrolysis of the azlactone to the dehydropeptide. This pro-
cedure was followed with the chloroacetylated derivatives of norleucine, valine, and
α-aminobutyric acid, and no difficulty was encountered in obtaining the corresponding
dehydropeptide. The compounds were recrystallized from either water or ethyl ace-
tate. Acetyldehydronorleucine: m.p. 135°C.; N calc. 8.2, N found 8.1; acetyldehydro-
valine: m.p. 198°C.; N calc. 8.8, N found 8.6; acetyldehydroaminobutyric acid:
m.p. 159°C.; N calc. 9.7, N found 9.5.

The absorption curves in the ultraviolet for the series of acetylated dehydroamino
acids are given in Fig. 1. For comparison, the corresponding curves for 2 saturated

acetylated amino acids are included. Of the dehydropeptides studied, only acetyl-dehydroalanine possesses a specific absorption in the ultraviolet region investigated. The curves for acetyldehydroaminobutyric acid, acetyldehydrovaline, acetyldehydro-leucine, and acetyldehydronorleucine are nearly identical, and, although revealing a far greater degree of absorption than the saturated peptides, possess only a general absorption in the region of the spectrum studied. This is in agreement with the observations of Fruton, Simmonds and Smith (1) on the absorption of acetyldehydro-leucine.
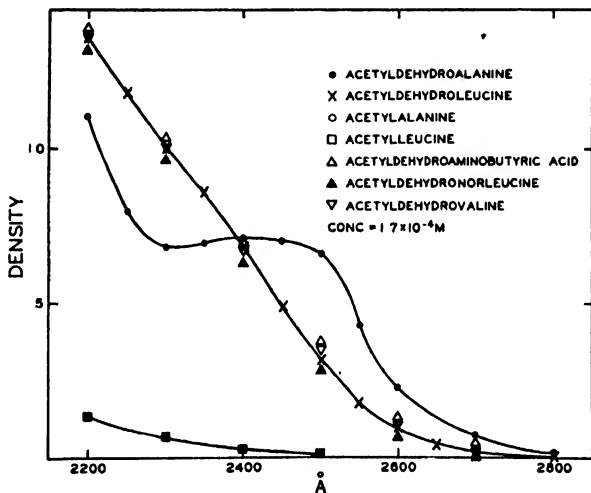
FIG. 1. Absorption curves in the ultraviolet of aqueous solutions of acetylated dehydroamino acids and amino acids. Cells with 1 cm. path.

The various acetylated dehydroamino acids were studied in digests with homogen-ates of rat kidney and liver according to the procedure described (2). The digests consisted of 1 cc. of freshly prepared tissue homogenate containing the equivalent of 333 mg. of tissue/cc., plus 2 cc. of 0.15 M borate buffer at pH 8.1 plus 1 cc., of either water or 0.025 M substrate solution. The hydrolysis of the substrates was measured by the ammonia evolved over the tissue controls. Up to two hours of incubation at 37°C., only acetyldehydroalanine yielded appreciable amounts of ammonia, at a rate approximating that reported earlier (2), namely, 1.8 micromoles substrate hydrolyzed /hr./mg. total N in the kidney extract, and 0.3 in the liver extract. Results with acetyldehydrophenylalanine, acetyldehydrotyrosine, acetyldehydroaminobutyric acid, acetyldehydrovaline, acetyldehydroleucine, and acetyldehydronorleucine were completely negative.

Many studies have shown that the enzymatic desamidation of glutamine in rat liver digests is accelerated by added pyruvic acid (6, 7). To see whether α-ketoiso-caproic acid could also serve in this connection, the keto acid was prepared in about 60% yield by the hydrolysis of acetyldehydroleucine in HCl. Twenty g. of pure acetyldehydroleucine were boiled under the reflux with 200 cc. of 2 N HCl for 2

hours. An aliquot of the digest was analyzed for free ammonia, and yielded nearly the theoretical amount (98%). On fractional distillation at reduced pressure, about 7 g. of α-ketoisocaproic acid was obtained. A part of the material was characterized as the 2,4-dinitrophenylhydrazone, which possessed an absorption maximum at 4300 Å at pH 12, and melted at 151°C.; N calc. 9.0, N found 8.6.

Rat liver digests were prepared as before (6, 7), containing 1 cc. of extract equivalent to 333 mg. fresh tissue, 1 cc. of veronal acetate buffer at pH 6.8, 1 cc. of either water or 0.014 M glutamine solution, and 1 cc. of either water or 0.025 M ketoisocaproate solution. After 4 hours of incubation at 37°C., the digest containing glutamine alone yielded 0.4 micromoles of amide ammonia N, and, in the presence of the added keto acid, it yielded 11.2 micromoles of amide ammonia N. The recovery of α-ketoisocaproic acid at the end of the incubation period in the presence or absence of added glutamine was of the order of 80%. Thus, like pyruvic acid, α-ketoisocaproic acid accelerates markedly the enzymatic desamidation of glutamine.

DISCUSSION

The striking difference (Fig. 1) in the character of the ultraviolet absorption curves of acetyldehydroalanine on the one hand, and of acetyldehydroaminobutyric acid, acetyldehydrovaline, acetyldehydroleucine, and acetyldehydronorleucine on the other, is indeed curious. Taken together with the susceptibility to enzymatic attack of the first-mentioned, and the lack of susceptibility of the latter group of compounds, the question is raised as to whether there may be any correlation between the 2 phenomena. Is the difference in molecular structure between the two sets of compounds, as revealed by their respective absorption spectra, a clue to their relative susceptibility to enzymatic attack? Not enough information is available to answer this, but it may be noted for future investigations. Since acetyldehydroalanine is hydrolyzed by an enzyme which has been designated as dehydropeptidase II (2) it, may be that this enzyme only involves peptides of dehydroalanine. Dehydropeptidases of the I series on the other hand, can apparently attack peptides of dehydrophenylalanine as well as of dehydroalanine. Whether such a peptide as glycyldehydroleucine will be metabolized remains for future investigation.

SUMMARY

Acetyldehydroaminobutyric acid, acetyldehydrovaline, acetyldehydronorleucine, acetyldehydroleucine, and acetyldehydroalanine were prepared and their absorption spectra and susceptibility to enzymatic hydrolysis in rat liver and kidney digests were studied. Acetyldehydro-

alanine possesses a specific absorption in the ultraviolet region and is readily metabolized. In contrast, the other compounds possess a general, high absorption in the ultraviolet and are not apparently metabolized. Acetyldehydrotyrosine and acetyldehydrophenylalanine are also not metabolized in these tissues.

α-Ketoisocaproic acid was prepared by the hydrolysis of acetyldehydroleucine in hot HCl. Like pyruvic acid, addition of this keto acid to a rat liver digest of glutamine at pH 6.8 produced an acceleration of desamidation of the glutamine.

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Turnover and Distribution of Phosphate Compounds in Yeast Metabolism[1]

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INTRODUCTION

The experiments to be reported here deal with the investigation of the phosphate metabolism of yeast during normal glucose fermentation and during the assimilation of growth-limiting amounts of nitrogen.

The variety of compounds studied is a point which deserves particular mention. It was desired to examine the rate of turnover of the nucleic acids, using the concentration of isotopic phosphorus as an indicator of this rate. From a high rate of turnover of a compound during a certain physiological process one might infer that this compound is important in the process. For this sort of logic to be valid, a minimal requirement would be the demonstration that the high rate of turnover is unique, or significantly greater than the turnover rates of other compounds. Accordingly, we have studied a number of classes of phosphorus-containing compounds simultaneously, so that their rates of turnover might be compared.

MATERIALS AND METHODS

A. General Methods and Activity Determinations

One strain of yeast was used—a pure culture of baking-type *S. cerevisiae*, numbered LK2G12 and sometimes referred to simply as K. This was grown in the following

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medium:

> Yeast extract—2.5 cc.
> Bacto peptone—5 g.
> $(NH_4)_2SO_4$—2 g.
> $MgSO_4$—0.25 g.
> $CaCl_2$—0.13 g.
> 50% Sodium lactate—7 cc.
> Glucose—60 g.
> Tap water—1 liter.

Forty-eight hour cultures were used exclusively.

Experiments were performed in the presence of 3–6% glucose. The final concentration of the P^{32} activity ranged from 1 to 10 microcuries/cc.; in a standard end-window Geiger-Müller counter (1) which was used for determining the radioactivity, one microcurie of phosphate assayed 2×10^5 counts/min. The final concentration of phosphate was adjusted to $M/60$. The final density of yeast in most experiments was about 50 mg./cc. on a wet weight basis. The yeast was washed twice in cold $M/60$ phthalate-NaOH buffer (pH 4.5) and suspended in phthalate buffer at pH 4.5 prior to dosage with phosphate. In experiments involving the use of exogenous nitrogen, this was added as $(NH_4)_2SO_4$, in an amount usually equal to about half the protein nitrogen of the yeast used (assuming the protein content of the yeast to range from 12 to 15% on a wet weight basis). It was demonstrated in separate experiments that this amount of added nitrogen would not permit appreciable multiplication to take place under our conditions, 5% budding being the most ever observed. Thus, it was certain that any effects of nitrogen on turnover rates were due to its assimilation and utilization rather than to the incorporation of phosphate which would automatically occur if a large number of new cells were formed. Biotin and calcium pantothenate were added to the experimental suspension in amounts of 0.025 γ/cc. to facilitate assimilation of the added nitrogen by the cells.

Most experiments were performed in conventional Warburg reaction vessels of 125 cc. capacity, the atmosphere being commercial nitrogen gas. In some experiments requiring larger samples, the suspension was placed in a 2-liter flask equipped with a 2-hole stopper carrying glass inlet and outlet tubes for gassing with nitrogen; the gas was used to stir the suspension during the experiment. By applying suction to the outlet tube through a side-arm flask, samples could be withdrawn when desired without disturbing the anaerobiosis of the remaining suspension. The temperature of the water-bath in all cases was 30°C.

Phosphorus was determined as inorganic phosphate by the method of Lohmann and Jendrássik (2), with the exception that Pictol (Mallinckrodt) was substituted for the usual reducing agents. Wet digestions for the determination of total phosphate were performed by heating samples in large Pyrex tubes with 1.5 cc. of 10 N H_2SO_4, a few drops of Superoxol being added near the end of the digestion to clear the solution. Any pyrophosphate which might have been formed during digestion was hydrolyzed by adding several cc. of distilled water and boiling vigorously for a few minutes. The optical density of the reduced phosphomolybdate was compared with that of a standard in the Klett-Summerson photoelectric colorimeter, using filter 66.

Samples for assay of radioactivity were neutralized to the phenolphthalein end point with concentrated NaOH, diluted appropriately, pipetted into small watch-

glasses, and evaporated to dryness under an infra-red lamp. They were assayed using a conventional Geiger-Müller counter tube with an end window, the counting circuit having a scale of 64. The counts actually observed generally ranged from a few hundred to a few thousand counts/min., with a background of about 30 counts/min. All counting was of such duration that the calculated statistical deviation was less than 3%. For weak samples with a higher statistical error the actual systematic error is indicated. In no instances was it necessary to apply corrections for self-absorption.

B. Fractionation of Yeast

The method of chemical fractionation used was selected after examination and trial of a number of procedures available in the literature, drawn primarily from studies on phosphate composition of animal tissues (3, 4). The final scheme was based
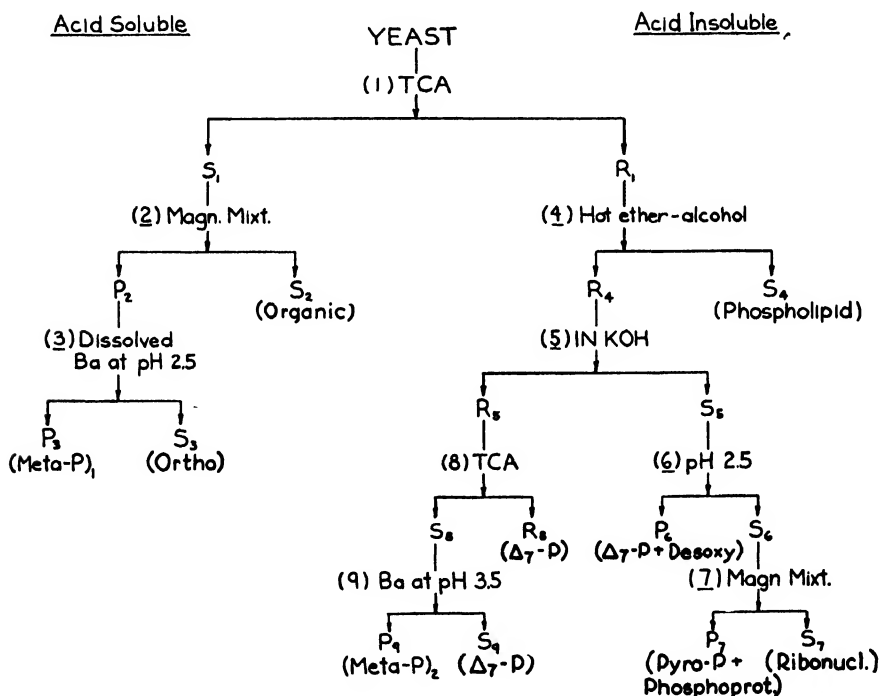
FIG. 1. General fractionation scheme for yeast phosphate

mainly on the method of Schmidt and Thannhauser (4) because it was required to separate the two types of nucleic acids gravimetrically to determine their specific radioactivity. It may be noted that in yeast cells the distribution turned out to be sufficiently unlike that observed in mammalian muscle or liver so that many questions arose regarding the purity of fractions obtained. The procedure finally adopted, while admittedly arbitrary in part, represented a reasonable combination of existing

methods appropriate for the organism used and for the purpose of these studies. The difficulties created by the use of this scheme will become evident in what follows.

The fractionation scheme is shown diagrammatically in Fig. 1. The sample of cell suspension, which had been incubated for the desired period in the presence of P^{32}-labeled inorganic phosphate and glucose (with or without a nitrogen source), was immediately centrifuged in the cold (0°C.). The cells were washed twice with ice-cold phthalate buffer (pH 4.5). (Separate experiments showed that this sufficed to remove all adherent radioactive material.) The cells were then suspended in about 10 volumes of cold 5% trichloroacetic acid (TCA) (sample for total P), and this suspension was kept for one hour at 3°C. After the cells were separated from the extract by centrifugation, they were briefly resuspended in a small portion of cold 5% TCA, and again centrifuged. The wash was combined with the main TCA extract, which was then brought to a definite volume with 5% TCA. This supernatant (S_1) contained all the "acid-soluble" phosphate.

1. DETAILED FRACTIONATION OF ACID–SOLUBLE PHOSPHATE

An aliquot of supernatant (S_1) was treated with Mg^{++} at alkaline pH to precipitate the inorganic orthophosphate resulting in fraction P_2 (5).

(Example: To 10 cc. extract were added 0.6 cc. concentrated NH_3 and 1 cc. of 15% $Mg(NO_3)_2 \cdot 6H_2O$. The mixture was alkaline to phenolphthalein. It was stirred vigorously with a small stirring rod, and left in the refrigerator for several hours. The precipitate was spun down, and washed twice with 3-cc. portions of dilute NH_3 (1:10). It was then redissolved in 1 cc. of 1 N HCl and 9 cc. of water.)

This left "organic" phosphate in solution (S_2). The precipitate P_2 was found to contain not only inorganic orthophosphate but also a considerable quantity of a compound whose phosphate could be converted to orthophosphate by hydrolysis for 7 mins. in 1 N HCl in a water-bath at 100°C. (Any phosphate compounds behaving in this fashion will hereafter be termed "7-min. hydrolyzable" or "7-min. phosphate," symbolized by Δ_7.)

It was shown that this fraction was not pyrophosphate by its failure to precipitate with Cd^{++} at pH 4.5 (6). Furthermore, a known quantity of pyrophosphate added as a carrier was recovered by Cd^{++} precipitation quantitatively, e.g., no additional phosphate in excess of that added was obtained.

It seemed plausible from a study of the literature (7, 8) to suppose that such a hydrolyzable fraction might be inorganic metaphosphate. To test the supposition that the labile fraction was in actuality metaphosphate, Ba^{++} was added to the solution obtained by dissolving the magnesia precipitate (P_2) in 0.1 N HCl, and the pH was brought to 2.5 with strong acetate buffer.

(Example: 10 cc. of redissolved P_2 adjusted to pH 2.5, 2 cc. of saturated $BaCl_2$ added.)

The characteristic gelatinous precipitate of barium metaphosphate appeared. A confirmatory test with toluidine blue gave the expected metachromatic reaction (9). All phosphate in the barium precipitate was 7-min. hydrolyzable. It appeared, on the basis of these tests, that the labile phosphate accompanying the orthophosphate

could be ascribed entirely to metaphosphate. MacFarlane (7) demonstrated that similar barium-precipitable material obtained from yeast had percentages of phosphorus and barium which agreed with the theoretical values to be expected for barium metaphosphate.

The precipitation was carried out at a pH of 2.5, rather than at the higher pH (4.5) recommended for maximum yield, to insure a high purity for the isolated metaphosphate. This was necessary because it was desired to determine its specific labeled (P^{32}) content, and contamination with other phosphate, particularly the orthophosphate of high specific activity, had to be minimized. About 80% of the total metaphosphate originally precipitated with the magnesia mixture (P_2) was recovered in the barium precipitate at pH 2.5 (P_3).

The supernatant (S_3), containing about 20% of the total metaphosphate and all of the orthophosphate from the original precipitate (P_2), could be used for estimation of the specific P^{32} content of the orthophosphate. A correction for the contaminating metaphosphate was applied after P^{32} assay of the purified metaphosphate (P_3).

The supernatant (S_2) remaining after the original magnesia precipitation should have contained only organic phosphate. However, an unexpectedly large percentage of this phosphate was found to be 7-min. hydrolyzable. The amount seemed excessive on the basis of adenosine triphosphate content expected; indeed, only a fraction of it could be ascribed to nucleotide phosphorus on the basis of spectrophotometric determination of nucleotides at 2600 Å (Beckman spectrophotometer). On adjusting the solution to pH 4.5 (acetate buffer) and adding Ba^{++}, a precipitate formed which contained about 50% of the total 7-min. phosphate in solution (S_2). About 70% of S_2 was found to be 7-min. hydrolyzable. The magnesia precipitation was known from separate experiments to give only partial precipitation of metaphosphate; and there were some indications that the amount precipitated was proportional to the amount of orthophosphate present. Hence, it seemed likely that much of the excess 7-min. phosphate remaining after magnesia precipitation was metaphosphate; its barium precipitability at acid pH supported this view.

The relatively high pH of 4.5 employed was dictated by the necessity for removing all such contaminating metaphosphate from (S_2) so that, in the ensuing assay of P^{32} content, a reliable value for the specific activity of the organic acid-soluble phosphate could be obtained. At pH 4.5 a relatively minor fraction of the true organic phosphate was precipitated, while all metaphosphate could be demonstrated to have been removed. The barium precipitate contained only 7-min. phosphate, and appeared to be almost entirely metaphosphate.

2. ISOLATION OF PHOSPHOLIPIDE PHOSPHATE

The cell residue (R_1) resulting from the cold TCA extraction was suspended once in cold 95% alcohol and centrifuged. The residue was then extracted 3 times for 3 minutes each with hot 3:1 ether-alcohol mixture at 68–70°C.

(Example: A sample initially of about 2 g. wet weight was treated with 5 cc. of alcohol, and then extracted with 5 cc. of ether-alcohol 3 successive times.)

The cold alcohol and the hot extracts were combined into a solution (S_4) which contained the phospholipide and other fatty material.

3. DETAILED FRACTIONATION OF FAT–FREE ACID–INSOLUBLE
PHOSPHATE RESIDUE

The fat-free cell residue (R_4) was now suspended in 1 N KOH and kept at 37°C. for 24 hrs.

(Example: To yeast with initial wet weight of 10 g., 40 cc. 1 N KOH were added.)

This procedure hydrolyzes ribonucleic acids to mononucleotides (10). Desoxyribonucleic acids remain virtually intact, while phosphoproteins are split to protein and inorganic orthophosphate (11 . On centrifuging the suspension after 24 hours, there were obtained fractions (R_5), the "KOH residue," and (S_5), which contained all phosphate originally present as nucleic acid phosphate and phosphoproteins.

Solution (S_5) was acidified with TCA and HCl following the method of Schmidt and Thannhauser (4). There resulted a precipitate (P_6) which presumably contained only desoxyribonucleic acid phosphate. Solution (S_6) was made alkaline to phenolphthalein and treated with magnesia mixture to precipitate orthophosphate (P_7), which should have comprised the phosphate present initally as phosphoprotein. The supernatant (S_7) should have contained only ribose nucleotide phosphate.

The rather bulky KOH residue (R_5) was found to contain phosphate, virtually all of which was 7-min. hydrolyzable. The residue was extracted with 10% TCA for 90 mins. at room temperature (*ca.* 25°C.). This removed much of the phosphate (S_8) and left some in the residue (R_6). The extract (S_8) was treated with Ba^{++} at pH 3.5 (acetate buffer), which resulted in the appearance of a precipitate (P_9). Supernatant (S_9) still contained an appreciable amount of phosphate.

Treatment of the fat-free acid-insoluble residue R_4 with 1 N KOH, followed by fractionation with acid and subsequent precipitation with magnesia mixture in alkaline medium, should have achieved clean separation of the ribose nucleotides, desoxyribonucleic acid, and phosphoprotein phosphate, assuming these to be the only kinds of phosphate present. Examination of the fractions obtained disclosed the failure to achieve complete purification.

Fraction (P_6), for example, should have contained only desoxyribonucleic acid phosphate. However, analysis revealed that about 80% of the phosphate present was 7-min. hydrolyzable—a result which could be explained only by the presence of a contaminant. The possibility that the labile phosphate could have resulted somehow from the preceding action of the KOH on desoxyribonucleic acid, while not very great, was considered and tested. In a known sample of desoxyribosenucleic acid, a slight effect was obtained by incubation with 1 N KOH for 24 hrs. at 37°C. The increase in 7-min. phosphate over that present initially was 6% of the total phosphate. Even treatment with 1 N KOH at 100°C. for 3 hours failed to give an increase of 7-min. phosphate greater than 16% of the total phosphate. Thus, at most 1% of the original 80% of 7-min. phosphate in (S_5) could possibly be ascribed to the KOH treatment ($0.06 \times 20\% = 1.2\%$).

The phosphorus "contaminant" which constituted most of (P_6) did not appear to be metaphosphate, pyrophosphate, or nucleotide phosphate; for a precipitate could be obtained neither at acid pH with Ba^{++} or Cd^{++}, nor at alkaline pH with magnesia mixture.

Obviously the specific activity of such an impure sample would have little significance with respect to turnover of desoxyribonucleic acid. As a guide in the further purification, the method of isotope dilution was tried (12). In this procedure, the process of purification is repeated (*e.g.*, successive isoelectric precipitations), usually in the presence of added inactive compound as carrier, until a constant specific activity is attained; it is usually assumed that the final fraction resulting is pure.

The specific activity of the crude fraction was 12 cts./min./γ of phosphorus. Inactive desoxyribonucleic acid was dissolved in the solution, bringing the activity down to 6.8 cts./min./γ. The pH was now brought to 2.5 with acetate buffer, and the resulting precipitate (the phosphorus of which represented only about two-thirds of the carrier added) assayed for radioactivity. The specific activity was now 6.27 cts./min./γ. Two more reprecipitations, resulting in a final recovery of only about 10% of the carrier added, gave substantially the same value, 6.17 and 6.28. Thus, it was to be expected that the sample was now pure desoxyribonucleic acid. Analysis revealed, however, that little desoxyribonucleic acid was present, and that the percentage of impurities had hardly been altered by the successive precipitations. It was conjectured that the impurities had formed a solid solution with the desoxyribonucleic acid precipitate. In this case the quantity of impurity would depend more on the distribution of one solid phase between the other solid phase and the solvent than on its solubility in the solvent alone.

It seemed likely that the large amount of protein present in the fraction might be contributing to the difficulties of purification. An attempt was made to remove as much as possible of this material by isoelectric precipitation at pH 4.65 (acetate buffer). A copious precipitate was obtained. The supernatant contained about 50% of the 7-min. phosphate, and all of the more difficultly hydrolyzable phosphate, which was identified as desoxyribonucleic acid. The procedure adopted for separating the 7-min. and the desoxyribonucleic acid phosphate was to hydrolyze the former to orthophosphate, and to precipitate the orthophosphate with magnesia mixture. The two components could now be assayed for radioactivity. The protein precipitate, which contained the balance of the 7-min. phosphate, was also assayed; the value did not differ significantly from that obtained with the 7-min. fraction of the supernatant, as was to be expected.

A specific identification of the desoxyribonucleic acid by the usual methods was rendered difficult by the large number of interfering substances present in the fraction. The diphenylamine reaction of Dische (13) was tried without success; a bright green color was observed instead of the expected blue. This difficulty persisted even after deproteinization. However, a method involving the reaction with cysteine, developed by Stumpf (14), gave excellent results on several trails, and was unaffected by the presence of the interfering substances which obviated the diphenylamine reaction.[4] Using this reaction, it was possible to show that the amount of desoxyribonucleic acid in the fraction checked closely with the value calculated from the amount of the non-7-min. phosphate. It should be noted that the amount of protein or protein split products still in solution after deproteinization was sufficiently great to render an accurate determination of nucleic acid by means of its ultraviolet

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[4] We are indebted to Dr. P. K. Stumpf for making the details of this procedure available to us in advance of publication.

spectrum impossible. In attempts to precipitate the nucleic acid with La^{+++} (15), large quantities of 7-min. phosphate were carried down with the precipitate.

The 7-min. component of (P_6) has not been conclusively identified as yet. However, after many compounds had been eliminated as described above, it was conjectured that a ribose phosphate might be involved. The orcinol reaction (16) was, therefore, carried out on an aliquot of the deproteinized fraction, together with a determination of 7-min. phosphate. The amounts of ribose and the 7-min. phosphate, expressed in molar quantities, were equal within the precision of the assay (0.49 ± 0.03 μM in the aliquot taken), thus lending the hypothesis support.

Passing on to solution (S_6), further complications appeared when the orthophosphate arising from phosphoprotein was precipitated with magnesia mixture. The precipitate (P_7) was found to contain 67% as the ubiquitous 7-min. phosphate. The contaminant was identified as inorganic pyrophosphate by means of its characteristic precipitability with Cd^{++} at pH 4.5 and with Zn^{++} at pH 6.5.

The supernatant (S_7) contained no labile phosphate. However, it proved to be impossible to determine ribose in this fraction with the orcinol reaction. The dark brown color, which was obtained, even at high dilutions and using the utmost precautions regarding purity of reagents and freshness of reagent solutions, pointed to the presence of some interfering compound or compounds (17). A spectrophotometric analysis in the ultraviolet, however, yielded a typical nucleic acid absorption curve. The amount of ribonucleic acid present, calculated from the optical density at 2600 Å corrected for nonspecific absorption, agreed satisfactorily with the amount calculated from the phosphorus content of the fraction.

4. DETAILED FRACTIONATION OF KOH RESIDUE (R_5)

The existence of an insoluble residue after prolonged treatment with KOH, which appears characteristic of yeast in contradistinction to animal tissues, was not entirely unexpected, in view of the large amount of alkali-resistant polysaccharide known to be present (18). What was surprising was the high percentage (27–39%) of phosphorus found in this fraction. The residue was analyzed first for 7-min. phosphate, in view of our experiences with the other fractions. Virtually all of the phosphate of the fraction fell into this category.

About 85% of the phosphate in the fraction could be extracted either by prolonged incubation with distilled water in the cold or by extraction with 10% TCA for 90 mins. at room temperature. An extract was prepared in the latter way (S_8), and Ba^{++} was added after the pH was brought to 3.5 with acetate buffer. A characteristic metaphosphate precipitate was obtained. The phosphate in this precipitate was almost entirely 7-min. hydrolyzable, and comprised 55–60% of the phosphate of R_5. It exhibited a typical metachromatic reaction with toluidine blue.[5]

Supernatant (S_9) contained, in addition to a small amount of metaphosphate not removed by precipitation at pH 3.5, an additional fraction which is as yet unidentified. R_6 was not easily extracted from the copious residue and, apart from activity measurements, no further studies on this fraction were made.

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[5] These tests were conducted in collaboration with Dr. J. M. Wiame, who also noted recently the appearance of an acid-insoluble fraction of metaphosphate in yeast.

EXPERIMENTAL RESULTS

A. Analytical Data

The distribution of yeast phosphate compounds in 48-hour cultures incubated for one hour in glucose-phosphate medium is shown in Table I. These data are in good agreement, wherever comparison is

TABLE I

Phosphate Distribution in the Yeast Cell

Nature of fraction	Type of phosphate	Total P per cent
Acid-soluble	Orthophosphate	9
	Metaphosphate	19
	Organic phosphate	10
Acid-insoluble	Phospholipide	3
	Phosphoprotein	2
	Pyrophosphate	4
	Nucleic acid	22
	Metaphosphate	17
	Unidentified [a]	11
	Unidentified [b]	4

[a] Associated with acid-insoluble metaphosphate.
[b] Associated with nucleic acid fraction.

possible, with the results of MacFarlane (7), who carried out the most thorough previous study of the phosphate distribution of intact yeast cells.

When the cultures were supplied with sufficient nitrogen in the form of ammonium sulfate to permit assimilatory activity but not appreciable cell division, the composition of the various fractions changed in the manner shown in Table II. The most conspicuous change occurred in the fraction labeled "KOH residue," which contained the acid-insoluble metaphosphate. While the changes in all other fractions upon addition of nitrogen lay between 10 and 25%, the residue increased by more than 50%. The importance of this change is accentuated by the fact that, even without nitrogen, this fraction was large, constituting 28% of the total phosphorus of the yeast cell, while, in the presence of nitrogen, it increased until it assayed 39%. Discussion of the significance of these figures will be deferred to a later section of this paper, in which the data on the specific P^{32} activity of the various fractions are considered.

TABLE II

Amount of Phosphate in Various Fractions of Yeast

The figures are in mg., based on the analysis of 10-g. (wet weight) samples of yeast after 3 hours under anaerobic conditions in the presence of 6% glucose and $M/60$ KH_2PO_4. The tracer phosphate was added at the end of the first hour. Samples with nitrogen were provided with $(NH_4)_2SO_4$ corresponding in amount to 50% of the protein nitrogen content of the yeast.

Nature of fraction	(1) Without nitrogen	(2) With nitrogen	$\frac{(2)}{(1)}$
Acid-soluble			
Orthophosphate	3.2	4.0	1.25
Metaphosphate[a]	6.6	7.3	1.10
Organic[a]	5.3	4.6	0.87
Acid-insoluble			
Phospholipide	1.2	1.1	0.92
Phosphoprotein and pyrophosphate	3.2	2.5	0.78
Ribonucleic acid	9.1	7.5	0.82
KOH residue[b]	10.5	16.0	1.52

[a] These fractions have been corrected for the metaphosphate remaining in the organic fraction after magnesia precipitation.

[b] 60% of this fraction consists of acid-insoluble metaphosphate whose specific activity is higher than that of the crude fraction.

In addition to the summary of significant results afforded by Tables I and II, it is of interest to present data which show how the various fractions behave analytically throughout an experiment. Such data are exemplified in Table III, which gives the phosphorus contents of the various fractions in mg., referred to samples of yeast of wet weight 10 g., at intervals throughout an experiment. Most of the fractions show no very remarkable changes in magnitude during the course of the incubation, either with or without nitrogen. The exception is the residue or acid-insoluble metaphosphate, which, in the presence of nitrogen, increases by more than 50%—a change also reflected in the data of Table II.

B. General Pattern of Phosphate Turnover

The results of the radioactivity assays in a typical experiment are exhibited in Table IV. The phosphate turnover is referred in all cases

to the P^{32} content of the exogenous labeled phosphate. Thus, the figure of 9.9% for orthophosphate in column (1) means that the specific activity of this fraction is 9.9% of the specific activity of the exogenous phosphate. In Table V, values for the specific activities of various fractions as a function of time are given.

The level of equilibration with the exogenous phosphate rises markedly in the presence of exogenous nitrogen. In most fractions this increase in turnover was accompanied by relatively small decreases in the total amount of the fraction (12% and 18%). In the case of metaphosphate, it was accompanied by a 50% increase in the amount of phosphate in the fraction. While the effect of nitrogen in increasing per cent turnover is greater for nucleic acid, the factor of increase for the number of micrograms of phosphorus turning over in each fraction is considerably greater for the acid-insoluble metaphosphate.

TABLE III

*Phosphate Distribution in Yeast as a Function of Time
with and without Exogenous Nitrogen*
(mg. P./10 g. wet weight)

Time	Inorg.	Meta[a]	Organic[a]	Acid-Sol	Phospho-lipide	Ribo-nucl.	P.P.[b]	"Desoxy"	KOH Res.
(*min.*)	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)
90	2.970	3.730	7.600	12.600	0.965	8.620	2.570	1.580	10.500
120	2.630	3.220	7.490	13.000	1.120	7.170	2.510	1.975	9.800
150	2.780	3.320	6.840	11.950	0.980	7.820	2.590	1.330	(6.850)
180	3.170	4.260	7.550	12.900	1.210	9.100	3.240	1.885	10.500
+N									
90	4.430	5.850	6.360	15.100	1.190	8.250	2.590	1.840	10.900
120	3.770	3.640	6.210	12.500	0.980	8.470	2.650	1.640	11.500
150	4.050	5.030	5.670	12.450	1.000	6.580	2.590	1.870	15.100
180	3.950	5.290	6.610	15.100	1.050	7.450	2.520	1.890	16.000

[a] Not corrected for meta-P in organic fraction (col. 3).
[b] Phosphoprotein plus pyrophosphate.

TABLE IV

Equilibrium Values for the Various Phosphate Fractions
with Nitrogen and without Nitrogen

The figures represent per cent equilibration with external labeled phosphate attained at the end of 3 hours of anaerobic incubation in 6% glucose and $M/60$ KH$_2$-PO$_4$. The tracer phosphate was added at the end of the first hour.

Nature of fraction	(1) Without nitrogen	(2) With nitrogen	$\frac{(2)}{(1)}$
Acid-soluble			
Orthophosphate	9.9	39.4	4.0
Metaphosphate[a]	0.0 (<0.03)	0.0 (<0.03)	—
Organic[a]	5.9	22.0	3.7
Acid-insoluble			
Phospholipide	3.3	14.3	4.3
Phosphoprotein and pyrophosphate	2.5	12.3	4.9
Ribonucleic acid	0.9	7.1	7.9
KOH residue[b]	6.2	33.9	5.5

[a] Corrected for metaphosphate not precipitated with magnesia mixture.
[b] Contains 60% of its phosphate as acid-insoluble metaphosphate.

Another striking result is the absolute lack of turnover in the acid-soluble metaphosphate with or without nitrogen. The complete absence of radioactive phosphate in this fraction is the more remarkable in that it was isolated from a fraction with the highest specific activity, the inorganic orthophosphate, so that it had a high probability of being contaminated with radioactive material. It appears that the metaphosphate in yeast is not only heterogeneous with respect to acid solubility, but is physiologically differentiated into at least two types (19).

The activity data obtained from the desoxyribonucleic acid and its 7-min. hydrolyzable contaminant have not been included in the tables, since they are not quite so extensive as the data for the other fractions. However, it may be noted that an activity for the desoxyribonucleic acid was obtained, which was nearly identical with that of the corresponding ribonucleic acid fraction, and much lower than that of the fraction as a whole, which showed an equilibration of about 19%. The 7-min. component of the fraction is, therefore, of fairly high activity

and turnover. The calculated specific activity (ct./min./γ) for the purified desoxyribonucleic acid phosphate in fraction D 120 was 3.22, the corresponding value for the ribonucleic acid phosphate was 3.27 (*cf.* Table V). The close correspondence between the desoxyribonucleic acid and the ribonucleic acid values is suggestive of the often-postulated relations between them (24).

The separation of the phosphoprotein and pyrophosphate fraction resulted in two components with comparable values of specific activity, *i.e.*, for sample 150+ N the value for pyrophosphate was 9.7 ct./min./ γ and for phosphoprotein 7.00 ct./min./γ.

In the present set of experiments, time curves were obtained for all fractions. The pattern which they revealed is exemplified in Fig. 2 in which are plotted the relative specific activities (fractional equilibrations— *i.e.*, figures defined in the same way as those of Table IV, but

TABLE V

Specific Activities[a] of Various Phosphate Fractions as a Function of Time (expressed in cts./min./γ P)

The specific activity of exogenous phosphate was 68.6 ct./min./γ P. To calculate the per cent equilibration as given in Table IV, divide the figures shown by 68.6 and multiply by 100.

Sample	Inorg.	Organic[b]	Ribonucl.	P.P[c]	Phospho-lipide	KOH residue
min. 90 (No N)	5.97	2.80	0.23	0.49	0.84	1.43
120 (No N)	6.78	3.55	0.38	0.79	1.29	3.22
150 (No N)	7.31	4.12	0.55	1.22	1.84	3.97
180 (No N)	6.74	4.04	0.63	1.73	2.26	4.24
90 (+ N)	23.9	6.00	1.44	2.51	4.00	7.57
120 (+ N)	28.8	9.05	3.27	4.75	6.76	13.0
150 (+ N)	26.9	12.4	4.30	7.43	8.60	17.9
180 (+ N)	27.0	15.1	4.83	8.41	9.82	23.2

[a] Standard deviation in all cases < ±5%.

[b] Corrected for metaphosphate not precipitated with magnesia mixture.

[c] Phosphoprotein plus pyrophosphate.
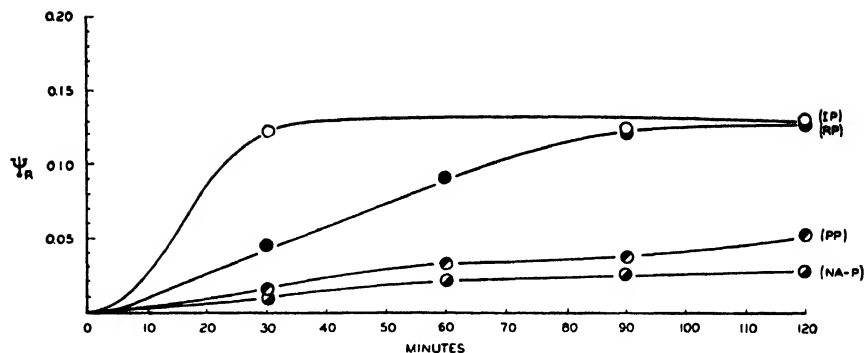
Fig. 2. Appearance of labeled phosphate in various fractions. Ordinate: Specific activity relative to exogenous phosphate (ψ_r). Abscissa: Time (mins.). I P = "Inorganic" phosphate. R P = KOH—Residue phosphate. P P = Pyrophosphate and phosphoprotein. N A P = Ribonucleic acid phosphate.
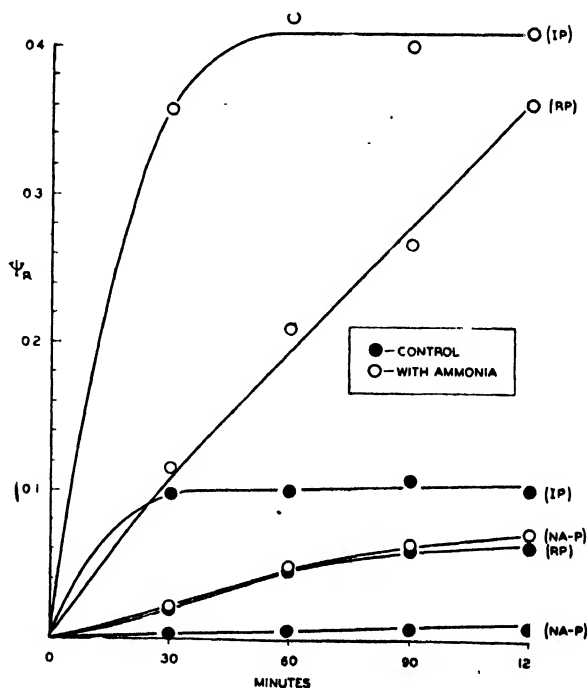
Fig. 3. Effect of exogenous nitrogen on phosphate turnover. Ordinate: Relative specific activity referred to exogenous phosphate (ψ_r). Abscissa: Time (mins.). Symbols have same meaning as in Fig. 2.

expressed as decimal fractions instead of percentages) of the various fractions during incubation with glucose-phosphate. The most important feature of these curves is the failure of any fraction to reach complete equilibrium with exogenous phosphate. In most of the cases, there is no indication that such equilibrium would be reached no matter how long the experiment were to be prolonged, for the curves have clearly leveled off before the end of the experiment. The attainment of the asymptotic or saturation value in the case of inorganic phosphate is quite early, being achieved in 30 mins. The rate of equilibration is less in all fractions than in the inorganic orthophosphate, and only the residue phosphate (the KOH or metaphosphate-containing residue) reaches a maximum value as high as that of the inorganic phosphate.

It might have been supposed that uptake of phosphate by the cell did not represent a true turnover, but merely a transient phenomenon due to the removal of the cells from their medium, washing, and subsequent resuspension in phosphate buffer. To test this, experiments were performed in which the labeled phosphate was added to the suspension some time after glucose and unlabeled phosphate had been added—a time sufficient to permit the supposed transient phenomenon to be completed. The time curves of activity, however, reckoned from the time of addition of P^{32}, were the same as those obtained when the tracer was added together with glucose and unlabeled phosphate. (The small lag when tracer is added at zero time appears to be connected with the mechanism of phosphate entrance, and will be discussed in a later paper.) It is clear from this finding that a true turnover is involved, and that the initial slopes of the time curves are a measure of the rate of this turnover.

In Fig. 3 and Table V the effect of nitrogen on the turnover-time curves is shown. The effect of nitrogen on the rate of equilibration is marked in all the fractions. It is most conspicuous, however, for the nucleic acid and residue fractions (cf. Table IV).

A feature revealed by this figure and not evident from data like those of Table IV is that the activity of the KOH residue in the presence of exogenous nitrogen, unlike all other fractions, is still increasing linearly at the end of the experiment, and gives no evidence of approaching a low saturation value.

It is impossible to say that the final values attained in these experiments are true equilibrium values. Cultures maintained for inordinately long periods under anaerobic conditions eventually take up more phos-

phate than those discussed above, but they also show signs of cell degeneration and autolysis. Complete equilibration under such conditions would have no biological significance; it would indicate only the known fact that dead systems attain complete equilibrium with their environment. The shape of the curves obtained in our experiments would argue that healthy cells, under the conditions described, do not completely equilibrate with their external environment (20).

An advantage of tracer experiments is that they offer an additional check (beyond that furnished by the analytical data) on the completeness of the material balance attained in the experiment. This check is given by the requirement that the total number of counts in a given sample of material shall be equaled by the sum of the counts of the various fractions into which that sample has been separated by the chemical procedures employed. The recoveries in all the experiments which we have performed have been good. Even in samples of very low

TABLE VI

Radioactivity Recovery Data for 180-Min. Samples in Presence of Exogenous Ammonia Nitrogen

(All data in ct./min./10 g. wet weight of yeast)

	Samples		Recovery
1	Organic	70,000	
2	Inorganic	107,000	
	Total acid-soluble (1)+(2)	177,000	
3	Acid-soluble (meas.)	185,000	$\frac{177000}{185000} \times 100 = 95\%$
4	Phosphoprotein pyrophosphate	21,200	
5	Ribonucleic	36,000	
	Total (4)+(5)	57,200	
6	Total (meas.)	61,000	$\frac{57200}{61000} \times 100 = 93.5\%$
7	"Desoxy"	24,400	
8	Phospholipide	10,300	
9	KOH residue	372,000	
	Total (3)+(6)+(7)+(8)+(9)	653,000	
	Total (meas.)	692,000	$\frac{653000}{692000} \times 100 = 94\%$

activity, where the probable error of the count is quite high, the sum of fractions and the total rarely have differed by more than 10%. Usually the agreement has been far closer. Examples of the kind of agreement obtained are given in Table VI. The data of this table pertain to

the same experiment as those of Table IV; the numerical values are cts./min., referred to a sample of yeast of 10 g. wet weight.

DISCUSSION

The experiments which have just been presented were undertaken to provide evidence bearing on the common supposition that nucleic acids are in some way causally related to the assimilation of nitrogen and formation of protein by living cells. The results furnish partial support for the affirmative view; the turnover of nucleic acids is, indeed, increased remarkably when the cells are assimilating nitrogen. However, the proviso of uniqueness—that the nucleic acid fraction shall be the only one to show a marked effect, or shall show it to an extent quantitatively far superior to all other fractions—is not fulfilled. Another fraction—the acid-insoluble metaphosphate—is stimulated to turn over by the addition of nitrogen to nearly as great a degree as the nucleic acid. Not only is this true, but the total amount of the fraction also increases sharply when nitrogen is available; the extent of the increase much exceeds that seen in any other fraction under any conditions which we have studied so far. Finally, this fraction, as well as the phosphoprotein, attains a specific activity considerably above that of the nucleic acid.

These findings raise the problem of the origin and physiological significance of this peculiar compound, and particularly of its relation to the nucleic acid. In this connection it is interesting to compare our findings with those of MacFarlane (7). This author, after removing the acid-soluble compounds from yeast, suspended the yeast in water and added sufficient NaOH to render the suspension neutral to phenolphthalein. Passage of material from the yeast into the solvent was immediately evident. The extract so obtained was found to contain nucleic acid, and a compound which MacFarlane identified as metaphosphate after a very careful study of its properties and elementary composition. MacFarlane supposed that the nucleic acid and metaphosphate existed as a complex; this was based in part on the finding that the metaphosphate so obtained was not found in yeast after autofermentation in the presence of toluene. MacFarlane also found metaphosphate in the acid-soluble fraction of the yeast.

In our experiments, the yeast was not extracted at neutral pH, but subjected to a long incubation with 1 N KOH. Since polymer metaphosphate is precipitated by such a concentration of alkali it seems likely that its presence, in our experiments, in the alkali-insoluble residue is due to such a precipitation and occlusion on the bulky residue of polysaccharide (which was washed with 1 N KOH rather than with

water). It is certainly possible that the metaphosphate was initially present as a complex with nucleic acid (or perhaps with protein), and that this bond was split by the incubation in strong alkali. However, neither our data nor those of MacFarlane are sufficient to establish that such a complex really exists.

Recalling the high energy of hydrolysis of the anhydride links in the hexametaphosphate, it is evident that such a possibility provides an instance to be added to the many known of the incorporation of a high-energy phosphate complex into a cell compound. A close association of nucleic acid and metaphosphate, with the possibility of a phosphate transfer occurring between them, might account for the quantitative identity of their relative turnover responses to nitrogen.

The finding that two kinds of metaphosphate occur, differing in their accessibility to acid and, more important, in their physiological functioning, is of importance for the problem of the interpretation of data obtained with tracer isotopes. Evidently the physiological differentiation would have been completely overlooked if we had chanced to select a fractionation procedure which would have isolated the two in one fraction. There is no guarantee that equally important *differentia* are not now being overlooked because of less sharply accentuated differences in accessibility or physical properties. One would moreover expect such physiological differentiations to occur frequently, in view of the many recent demonstrations (21, 22, 23) of structural isolation and semi-isolation of cell components on a microscopic scale. *It seems essential, therefore, to interpret data obtained from tracer experiments with great reserve until the fractions obtained have been reproduced by alternative methods of fractionation.*

Equally interesting is the finding that all phosphate fractions fail to equilibrate with the exogenous phosphate. This again suggests the existence of physiological and structural compartments in the cell, such that the contents of some of them are out of contact with others and with the cell's external environment under the conditions of our experiments. Such materials would not exchange with exogenous tracer. The fractions examined must then be regarded as mixtures of classes of chemically identical but physiologically quite distinct molecules. The mixtures of material which has completely equilibrated and material which has not exchanged result in fractions which appear to have equilibrated in part.

There are several fractions, some identified, some inconclusively identified, and some entirely unidentified, whose presence in the cell after acid extraction has as yet found no satisfying explanation. It might be argued that the pyrophosphate, for example, resulted from partial hydrolysis of hexametaphosphate, perhaps during the alkaline incubation; this is certainly a chemical possibility. However, the specific activity of this material differs from that of the two known metaphosphate fractions; and one would, therefore, have to postulate still a third kind of metaphosphate. This is admittedly not to be excluded *a priori*. It is also possible that the pyrophosphate might have been linked to the protein, as is suggested by the identity of its specific activity with that of the "phosphoprotein phosphorus." The experiments on phosphoproteins do not exclude either of two possibilities: that there exist pyrophosphate groups linked to proteins as well as orthophosphate groups; or that only pyrophosphate linkages occur in native proteins. In either eventuality, one might argue that drastic treatment in obtaining the proteins—*e.g.*, use of strong acids, or acid hydrolysis in obtaining amino acids—would have obscured the presence of the pyrophosphate linkage.

It was suggested, because of the finding that the ribose in the desoxy fraction matched the 7-min. phosphate, that the labile contaminant in this fraction might be a ribose phosphate. This might be invalidated if the ribose were in fact a contaminant from the ribose nucleotide fraction. However, this would require contamination with 10% of the ribonucleic fraction after careful washing of the desoxyribonucleic precipitate with buffer. If it be argued that the contaminant was occluded in the bulky protein-containing precipitate too well to be washed out, it is difficult to account for the appearance of ribose in the supernatant after the protein was subsequently removed by another acid precipitation. Moreover, if the contaminant were ribose nucleotide, some trace of Hg^{++}-precipitable material should have been detected; and we have shown that this was not the case. The failure of the ribose phosphate to appear in the acid-soluble fraction may again be explained by assuming that it was linked to some larger molecule.

Our experiences emphasize the difficulties inherent in studies of this kind, and the limitations of the tracer technique as used to investigate metabolic mechanisms. It is not easy to obtain pure and identifiable compounds. It is uncertain what their activities mean, except in particularly simple circumstances.

It seems reasonable to expect that the simultaneous use of two or more tracers (*e.g.*, C^{14}, N^{15}, and P^{32}) would assist in clarifying the interpretation of comparative activity data.

Finally, it is evident that some of our difficulties are inescapable. The property of living systems which makes them peculiarly interesting for tracer studies—the continual activity and turnover even in seemingly stable components of the cell—implies that it may be difficult to achieve a completely unambiguous correlation between turnover in a given compound and its participation in a certain metabolic or physiological process, without the manifestation of turnover in compounds which are irrelevant to that process. The cell will not cease exchanging phosphate in all its phosphorus-containing compounds simply because it is engaged in synthesizing proteins. One may observe only that marked differences in rate will occur, which will permit a choice of that compound the turnover of which bears on the process being studied. In deciding how much larger one effect must be than another there will of course be an irreducible minimum of arbitrariness.

Another difficulty which should be mentioned arises from the possibility of non-linear relations between metabolic processes, even fairly simple ones. Suppose the curve which relates the rate of protein synthesis to the necessary turnover of the compound which constitutes the protein-synthesizing machine rises and then levels off. Suppose also that the rate of turnover is normally high enough to be at the level part of the curve. This would mean that the machine usually runs at top speed even when there is no load—that the phosphate is always turning over, but the turnover is utilized only when nitrogen assimilation makes synthesis possible. Then the addition of nitrogen would have little or no effect on the observed turnover. The crucial compound would be overlooked in favor of some other, whose turnover is stimulated by nitrogen, not because it is involved in making protein, but because, for instance, the protein which is made acts upon this compound.

SUMMARY

The distribution and turnover of phosphorus-containing compounds in living yeast cells have been studied with the aid of exogenous inorganic orthophosphate labeled with P^{32} as tracer. Cells were incubated under anaerobic conditions in glucose and phosphate medium. They

were then fractionated by a modification of the method of Schmidt and Thannhauser to obtain the acid-soluble compounds, organic and inorganic, the phospholipides, the phosphoproteins, and the two varieties of nucleic acid. Each fraction was analyzed for its content of phosphorus, and assayed for its content of P^{32}.

Several compounds not usually encountered in animal tissues complicated the fractionation procedures. A large quantity of inorganic metaphosphate was found in the acid-soluble material. The phosphoprotein phosphorus was contaminated with pyrophosphate which had survived extraction by acid. The desoxyribonucleic acid fraction contained a large quantity of a labile phosphate compound, which preliminary work indicates may be a ribose phosphate. The polysaccharide residue, which did not dissolve in alkali after prolonged incubation, contained a large amount of metaphosphate, which was not originally extracted by acid.

The turnover indicated by the specific activity of P^{32} in the various fractions was studied as a function of time. The inorganic orthophosphate exhibited the most marked activity. The activity of all fractions reached saturation values far below those corresponding to complete equilibrium with the exogenous phosphate.

Ammonia nitrogen was added to the yeast suspensions in amount sufficient to permit assimilation but not appreciable cell division. Under these conditions the rate of turnover in all fractions was enhanced. The most marked effect was shown by nucleic acid and the acid-insoluble, or residue, metaphosphate. Almost all the fractions still approached saturation with respect to specific activity at values below complete equilibration with exogenous phosphate. The residue metaphosphate, however, increased linearly with time, and had given no signs of reaching saturation at the end of the experiment. The acid-soluble metaphosphate, in contrast, showed no appreciable turnover, either in the presence or absence of nitrogen.

The significance of these results is discussed, with particular emphasis on the possible relations between the nucleic acid and residue metaphosphate, and their function in protein synthesis.

Some of the difficulties involved in the interpretation of comparative specific activity data with regard to metabolic mechanisms are briefly considered.

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Phosphate Metabolism and the Dissociation of Anaerobic Glycolysis from Synthesis in the Presence of Sodium Azide[1,2]

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INTRODUCTION

It is well known that sodium azide (NaN₃) can prevent synthetic activities of various kinds, including such diverse processes as the assimilation of carbohydrate and embryogenesis. Clifton (3) has reviewed the data available bearing on the effect of azide as well as other agents on assimilatory processes in microorganisms.

The fact that azide at certain concentrations can prevent cellular syntheses which depend on aerobic metabolism can be correlated with the original observations of Keilin (4), who showed in some detail the ability of this compound to inhibit the cytochrome oxidase system. Of further interest is its capacity to prevent cellular synthesis coupled with anaerobic metabolism. Winzler (5) has shown that the anaerobic assimilation of glucose does not occur in the presence of NaN₃, nor can yeast take up nitrogen under these conditions (6). Furthermore, embryonic development in the frog, which can occur under anaerobic conditions or in the presence of sodium cyanide, is completely hindered by NaN₃ (7), as is the ability of yeast cells to utilize the energy of anaerobic glycolysis for enzymatic adaptation (8).

In view of the wide variety of synthetic reactions inhibited, it seems probable that NaN₃ immobilizes one of the primary energy sources of the cell. The elucidation of the mechanism of action of NaN₃ may provide an important clue to the nature of the link between the energy-

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generating mechanism of the cell and those reactions which utilize this energy for the synthesis of the various cellular components.

Following Lipmann's (9) suggestions concerning the role of energy-rich phosphate bonds in the storage of utilizable energy, it is plausible to suppose that inhibition of synthetic activity would result from interference with the formation of such phosphate bonds. Data exist which support this concept.

Thus, the observations of Krahl and Clowes (10) that 2,4-dinitrophenol (DNP) can inhibit cell division at concentrations which stimulate respiration may be correlated with the work of Ronzoni and Ehrenfest (11), who have shown that DNP caused a decrease in phosphocreatine in frog muscle. More recently, Hotchkiss (12) has reported on the ability of this nitrophenol to prevent phosphate uptake by yeast respiring in glucose medium. This author also remarks that azide in concentrations which are not inhibitory to the respiratory mechanism nevertheless prevent a net increase in the P content of yeast cells metabolizing glucose aerobically. Rothstein (13) finds that the presence of azide inhibits the redistribution of phosphate which normally occurs when yeast cells are incubated with glucose. However, the latter experiments were carried out under aerobic conditions at concentrations which could suppress respiration, so it is difficult to ascertain how much of the inhibition observed is due to the effect on the cytochrome system. · ,

Kalckar (14) pointed out that the availability of acceptors for the energy-rich phosphate generated by glycolysis could act as a regulating mechanism of glucose metabolism. Brockmann and Stier (15) explain the stimulation of glucose fermentation often observed with NaN₃ on the basis that this agent prevents the generation of energy-rich phosphate, a supposition suggested by experiments with radioactive tracers and inhibitors (1, 2). Brockmann and Stier (16) have employed this fact to devise a system which permits a more absolute determination of glucose-fermenting capacity than has been previously possible.

It was the purpose of the present research to examine in greater detail the nature of the interference with the phosphate metabolism of the cell and to attempt to localize that point in the phosphate cycle at which this interference took place.

To obtain direct evidence on the effect of azide on phosphate metabolism, recourse was had to the use of P^{32} as a tracer. In connection with localization of the azide effect, all experimental analyses of the problem reported have been limited to anaerobic conditions. There were several reasons why this procedure was employed. First, comparatively little is known about the relation between the aerobic pathway of oxidation and phosphate esterification, whereas our knowledge of the coupling between anaerobic oxidation and the entrance of P into organic combination is relatively precise. Secondly, metabolism in the presence

of oxygen at the usual tensions employed is a composite of the resultant activities of the aerobic and anaerobic pathways, and, to that extent, would be a more difficult situation to analyze, even if both components were perfectly understood.

MATERIALS AND METHODS

a. The yeast strain (KI) employed is a representative of *S. cerevisiae* and is one of those used in previous (17) experiments on enzymatic adaptation. Except in one instance specified, all the experiments were performed on 48-hour cultures grown at 30°C. in media described below in standing flasks.

b. The culture medium was made by adding the following to 1 liter of water: 4 g. of $(NH_4)_2SO_4$, 2 g. of KH_2PO_4, 0.25 g. each of $CaCl_2$ and $MgSO_4$, 7 cc. of 50% Na lactate, 2 cc. of Anheuser-Busch liquid yeast extract No. 3 (obtained through the courtesy of Anheuser-Busch & Co., St. Louis, Mo.), and 60 g. of glucose. The mixture was brought to a boil and then filtered.

c. Manometric measurements were made at 30.2°C. with standard Warburg apparatus. The anaerobic CO_2 production was determined by replacing the air with nitrogen.

d. Standard suspensions containing approximately 2 mg. dry weight of yeast/cc. were prepared from cells washed twice with cold $M/15$ KH_2PO_4 and brought to the desired density with the aid of a photoelectric colorimeter, the readings of which had been previously standardized.

e. Phosphate was analyzed by the method of Fiske and SubbaRow (18) and reducing sugar by the method of Folin and Malmros (19).

f. The inhibitors employed were all dissolved in $M/15$ KH_2PO_4 and adjusted to pH 4.5, with one exception, *p*-chloromercuribenzoate, which was used at pH 5.6. The rigid control of pH has been observed to be of crucial importance in these experiments, especially where interactions between the effect of various inhibitors[3] was being examined.

g. The assay of P[32] was accomplished, using the conventional type of end-window Geiger-Müller tube in a variation described elsewhere (20). A scaling circuit (scale of 64) and mechanical counter were coupled to the G.-M. tube in the usual manner. Aliquots of the radioactive solutions were brought to pH 8 with concentrated NaOH and pipetted onto watch glasses, which were matched for thickness and curvature. The samples were evaporated to dryness under an infrared lamp. The amounts of solid matter resulting never exceeded 10 mg./cm.[3] so that no self-absorption corrections were required. In most cases, samples were diluted until well within the counting range in which the correction for loss by coincidence of pulses was negligible (~ 3000 ct./min.). Coincidence corrections were made when necessary, using a correction curve constructed from standards assayed under the same conditions as the experimental samples (21). A rack made possible the placement of samples at various distances from the counting tube window. In many instances, samples with too high a

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[3] The sample of iodoacetamide employed was obtained through the kindness of Dr. L. Hellerman. We should also like to extend our appreciation to Dr. E. S. Guzmann-Barron for a supply of *p*-chloromercuribenzoate.

count were assayed at distances from the counter sufficient to drop the counting rate to an acceptable value. The correction factor for distance was determined by calibrations with a sample the counting rate of which permitted determination in all positions provided by the sample rack. Numerous control experiments showed that accuracy of sampling was such that reproducibility was better than 5%. Counting was carried out for periods long enough to limit statistical errors to less than 3%.

Experimental Results

a. The Effect of NaN₃ on P Turnover

Experiments were performed in which suspensions of yeast cells were allowed to ferment anaerobically a given amount of glucose in the presence of P^{32}-labeled phosphate, with and without NaN_3 in the medium. Without disturbing the anaerobiosis, samples were removed at intervals, the cells washed free of contaminating exogenous labeled phosphate, and assayed for P^{32} and total phosphate content according to the procedures previously described. Simultaneously, glucose was determined in the supernatant medium. Results typical of such experiments are given in the curves of Fig. 1. In the experiment shown, the suspending medium was $M/30$ in phosphate adjusted to pH 4.5, and the concentration of the azide in the experimental suspension was 2.5×10^{-3} M. The upper curves of Fig. 1 show the results of the sugar determinations. It is seen that no interference with glucose utilization occurred at the azide concentrations employed. In the lower curves, ψ_R, the ratio of specific activity (ct./min./γ P) of the internal phosphate to that of the exogenous phosphate, is plotted as a function of time. $\psi_R \times 100$ yields the per cent equilibration of the internal phosphate with that in the external medium.

At 120 minutes both suspensions had metabolized equal quantities of glucose. However, the azide-treated suspension exchanged only about 3% of its P content, whereas the control had, in the same period, exchanged to the extent of 26%. It appears quite definite from these results that azide interferes with the phosphate metabolism of the cell.

b. The Effect of Azide on the Internal Orthophosphate

Although the experiments described in the previous sections strongly indicate that one must look to the phosphate cycle for an explanation of the azide effect, they do not constitute conclusive evidence that this is the case. It can be argued that the presence of azide renders the cell

"impermeable" to phosphate. This explanation could be offered both for the inability to accumulate P from the external medium in the presence of azide, as well as for the absence of an exchange between inside and outside phosphate. A test of the permeability hypothesis was attempted by examining the effect of azide on the distribution
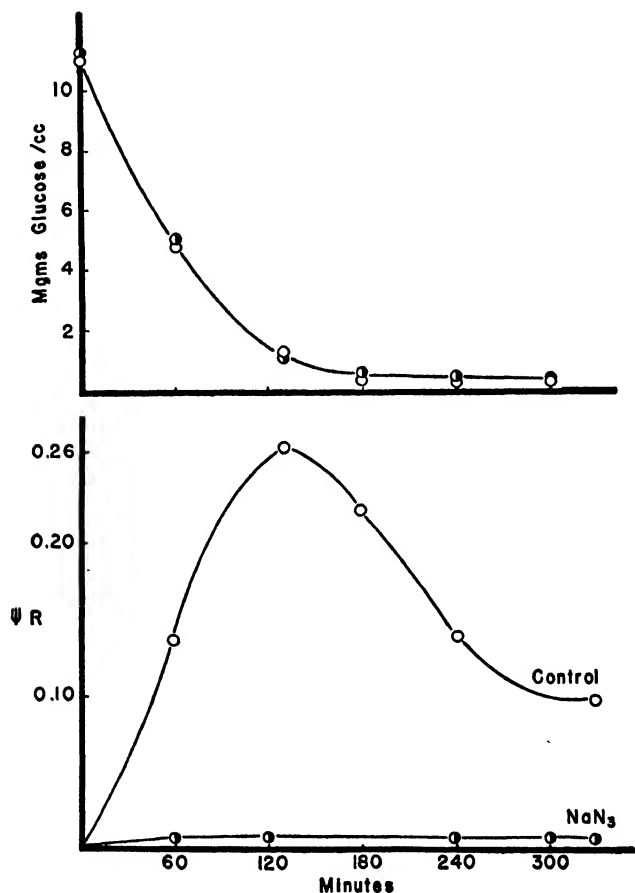
FIG. 1. The effect of sodium azide on phosphate exchange in anaerobically fermenting yeast cells. In the upper curves, the concentration of glucose remaining in the medium is plotted as a function of time. In the lower curves, the ratio (ψ_R) of the specific activity of the cellular phosphate to the specific activity of the exogenous phosphate is plotted for the same time intervals. The open circles indicate results obtained in the absence of NaN_3; the half-shaded circles refer to results obtained in the presence of NaN_3 ($2.5 \times 10^{-3} M$).
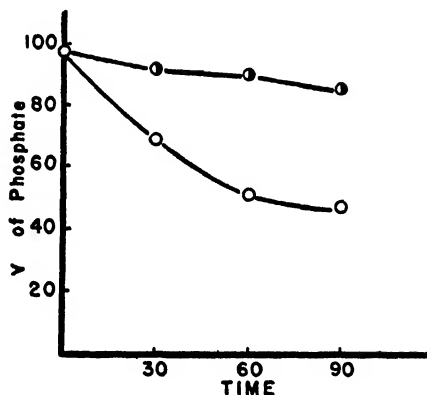
FIG. 2. The behavior of internal inorganic phosphate during anaerobic fermenta-
tion in a phosphate-free medium in the presence (half-shaded circles) of sodium azide
$(2.5 \times 10^{-3} M)$ and in its absence (open circles). Time is in mins.

of the internal phosphate during fermentation under conditions where
entrance of external phosphate was not involved. These experiments
were performed in a phosphate-free medium. Cells were suspended in
$M/15$ phthalate buffer at pH 4.5 and allowed to ferment glucose an-
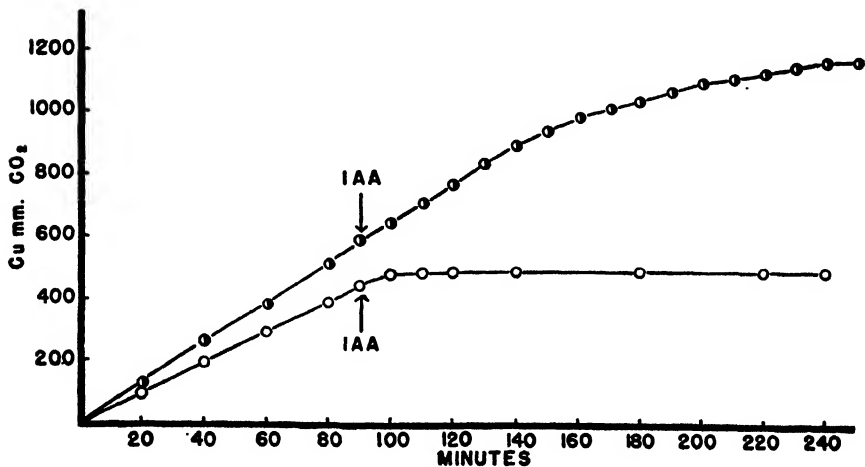
FIG. 3. The effect of NaN_3 on the sensitivity of anaerobic fermentation to mono-
iodoacetic acid $(2.5 \times 10^{-4} M)$. The half-shaded circles refer to fermentation in the
presence of NaN_3 $(5 \times 10^{-3} M)$, and the open circles to fermentation in its absence.
The arrows indicate time of addition of the monoiodoacetic acid.

aerobically in the presence and absence of 2.5×10^{-3} M NaN$_3$. Samples were removed at intervals for determinations of the ortho-phosphate content of the cells. The behavior of the orthophosphate in the acid-soluble portion in the course of such an experiment is given in Fig. 2. It is clear that a considerable portion of the internal inorganic phosphate disappears during fermentation, and that this drop is largely prevented by the presence of NaN$_3$. This difference in decrease of the orthophosphate cannot be explained by an azide interference with loss of phosphate to the environment. Analysis of the external medium at the end of the 90 minutes of fermentation revealed that the cells in both suspensions had lost 10γ of inorganic P to the outside.

Since all the phosphate in this experiment was intracellular, it is evident that "penetration" was not involved. One cannot by such experiments rule out the possibility that azide affects the permeability of cells to phosphate. However, factors other than permeability must be involved, since one must conclude from these results that azide can effectively prevent the flow of phosphate from the inorganic to the organic fraction which accompanies the fermentation of glucose.

c. The Effect of NaN$_3$ on Glycolytic Inhibitors of the Triosephosphate Dehydrogenase

It is evident that azide can interfere with the glycolytic mechanism in a manner such that the metabolism of glucose does not result in a normal esterification of inorganic phosphate. The question which must now be posed is: At what point or points in the cycle of carbohydrate metabolism can the action of azide be localized?

From what is known about the nature of the glycolytic cycle, there are only two reactions whereby inorganic phosphate can enter into organic combination anaerobically. One is the phosphorolysis of glyco-gen in which glucose-1-phosphate is formed. The other is the coupled oxidation and phosphorylation of 3-P-glyceraldehyde which results in the formation of 1,3-diphosphoglycerate. There are several reasons why it is not likely that the first step mentioned can be responsible for any major portion of phosphate esterification during the fermenta-tion of glucose. First, it would presuppose that the formation of glyco-gen is a necessary preliminary for the entrance of glucose into the glycolytic cycle. Furthermore, no net P esterification could result from this series of reactions, since one organic phosphate bond from ade-

nosine triphosphate would have to be mineralized for every ester link formed during the subsequent phosphorolytic cleavage of glycogen. This mechanism could not, therefore, explain why the presence of azide prevents *net* P uptake into the organic fraction.

On the basis of the above reasoning, it seemed reasonable to conclude that effects on the phosphorolytic step alone could not account for the phenomena observed in azide-treated cells. It seemed plausible to suppose, therefore, that this agent was affecting the glycolytic mechanism in such a manner as to permit the oxidation of the glyceraldehyde phosphate without a resultant phosphate esterification.

A further analysis of this suggestion could be gained by an examination of the properties of the coupled oxidative step in the presence of azide. It was reasoned that, in the presence of azide with resultant uncoupling of oxidation from phosphorylation, the fermentation of intact cells might become less sensitive to poisons capable of inactivating the oxidative enzyme, triosephosphate dehydrogenase. The investigations of Rapkine (22) showed this enzyme was one which depended on the intactness of its sulfhydryl groups for its activity. He demonstrated that agents which interacted with SH groups inactivated the enzyme. Among such agents may be cited iodoacetic acid (IAA), *p*-chloromercuribenzoate, phenyl mercuric acetate and iodoacetamide.

The first glycolytic poison to be examined was iodoacetic acid (IAA). A concentration of IAA was chosen 2.5×10^{-4} M) which, by preliminary experiments, was shown to produce complete inhibition of the fermentation within 10 mins. Fig. 2 shows the results of an experiment in which the effect of azide (0.005 M) on this inhibition was examined. The NaN_3 was put in the main compartment with the suspension, the IAA in one of two sidearms, and the glucose in the other. The final glucose concentration after tipping was 3%. After flushing the vessels with nitrogen and temperature equilibration, the glucose was tipped and the fermentation allowed to proceed. Subsequently, the IAA was tipped into the main compartment, which introduction is indicated in Fig. 2 by the arrows. It wil be noted that, in the control suspension, complete cessation of CO_2 evolution occurred in 10 mins. following the addition of the IAA. However, in the case of the suspension with NaN_3 no signs of inhibition were observed for the first 40 mins. Following this, a slow decline in the rate of fermentation was observed. It was not until 150 mins had elapsed after the addition of the IAA that a complete inhibition was finally achieved.

These results would appear to indicate that a marked change in sensitivity to a triosephosphate dehydrogenase inhibitor does occur on the addition of NaN_3, a finding which is consistent with the hypothesis that the interaction of NaN_3 with the P-esterifying mechanism of the glycolytic cycle is either at the triosephosphate dehydrogenase itself, or involves the product of its activity.

It was necessary to examine the possibility of a direct interaction between the azide and the monoidoacetic acid which might lead to the destruction of the latter. Attempts were made to detect such a reaction in various ways. The methods tested included manometric examination for the release of nitrogen which might be expected in a reaction involving azide. A colorimetric analysis for the presence of azide with $FeCl_3$[4] was also performed. No release of nitrogen was observed, and the amount of azide detectable with the $FeCl_3$ was not changed on incubation with IAA, nor could iodide or free iodine be found in mixtures of the two reagents after prolonged incubation. To facilitate the detection of the reaction if any existed, 0.5 M solutions of both IAA and NaN_3 were employed in these experiments.

The possibility that a direct interaction was occurring, undetected by the methods employed, was examined by using the yeast as a test system. If such a reaction were taking place it would be expected that previous incubation of the two agents together should lead to more effective protection by NaN_3 against the IAA inhibition than if the azide were put in the yeast suspension and then followed by the IAA. Furthermore, the effectiveness of the counteraction of azide against the IAA should increase with the length of the preincubation of the mixture. Such experiments were performed by placing the IAA and NaN_3 in the same sidearm and tipping after various periods. The results obtained in these instances were compared with those in which the two were kept in different sidearms and introduced separately. The protective action was measured by the time required to reach zero fermentation rate after the introduction of the IAA.

The results of these experiments are summarized in Table I. In the first 3 experiments, the IAA was added 20 mins. after introduction of the NaN_3. Protection for a period of approximately 150 mins. was observed. Expts. 9–11, inclusive, represent controls in which only IAA was added and permitted comparison with those in which the effect of azide was being studied. In Expts. 4–8, inclusive, the IAA and

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[4] H. B. Steinbach, private communication.

TABLE I

The Effect of the Order of the Addition on the Protective Action of NaN₃
on Monoiodoacetic Acid Inhibition of Fermentation

All these experiments were carried out anaerobically on yeast suspensions in the presence of 3% glucose in $M/15$ KH_2PO_4 at pH 4.5. All agents were dissolved in the same medium and the pH adjusted when necessary. The numbers in the last Col. give the time in mins. required to reach a zero fermentation rate subsequent to the addition of the IAA. In Expts. 4–8, inclusive, the two agents were added from the same sidearm and the numbers in parenthesis indicate the period during which the mixture was incubated before the addition to the yeast suspension. In all cases the final concentration of the NaN₃ was 5×10^{-2} M and that of the IAA 2.5×10^{-4} M.

Experiment no.	First addition	Second addition	Time to zero rate
			mins.
1	NaN₃	IAA	150
2	NaN₃	IAA	140
3	NaN₃	IAA	150
4	NaN₃ + IAA (20)	—	70
5	NaN₃ + IAA (60)	– ·	60
6	NaN₃ + IAA (60)	—	70
7	NaN₃ + IAA (120)	—	80
8	NaN₃ + IAA (120)	—	70
9	—	IAA	10
10	—	IAA	10
11	—	IAA	10

NaN₃ were incubated in the same sidearm for various periods of time and then added simultaneously. It is clear from a comparison with the results of Expts. 1–3 that, instead of increasing the protective action of the azide, this preincubation of the mixture of the two agents considerably decreased the extent of the phenomenon. The extent of the protection observed was in no way influenced by the length of the preincubation period. It seems likely that the decreased protection observed in these experiments is due simply to the fact that a little time is required before the presence of the azide influences the glycolytic system. Thus, when the two agents are added simultaneously, there is a short period during which the IAA is actually interacting with a normally functioning glycolysis. It is evident that these results are not consistent with the notion that the protective action of azide is due to a direct interaction with the IAA which leads to the destruction of the latter.

The effect of adding the azide subsequent to the IAA was also tested. In these experiments the azide was added 10 mins. after the IAA, and hence at the time when the IAA had achieved its complete inhibition. In no case was recovery of the fermentation observed on the addition of the azide. Thus, the order of the addition of these two agents has a profound influence on the protective phenomenon, it being completely absent if the glycolytic poison is added first.

Table II presents a survey of the effective range of NaN_3 concentra-

TABLE II

The Effectiveness of Various Concentrations of NaN_3 in Decreasing Sensitivity of Fermentation to Inhibition by Monoiodoacetic Acid

The numbers in last column indicate time interval between the addition of the IAA and the attainment of a zero rate of fermentation. See text for further details.

Molar concentration of NaN_3	Per cent of control QCO_2	Time to zero rate in minutes
0	—	10
.0001	102	10
.0002	104	10
.0005	111	20
.001	130	40
.005	108	150
0.01	85	190
0.05	63	230
0.10	58	240
0.25	51	30
0.33	49	20
0.50	40	10

tions for eliciting protection against IAA. In all the experiments recorded, the yeast were suspended in $M/15$ KH_2PO_4 at pH 4.5 and reagents were dissolved in this buffer and adjusted to the same pH when necessary. The proper amount of NaN_3 was put in with the yeast suspension and, after the establishment of anaerobiosis and equilibration, sufficient glucose to make a final concentration of 3% was tipped from one sidearm. The fermentation was allowed to proceed for 1 hour by which time an accurate determination of the rate could be made. The IAA (2.5×10^{-4} M final concentration) was then introduced and readings taken at 10 min. intervals until the rate fell to zero.

It is evident from Table II that, under the conditions of these experiments, concentrations below 0.001 M were not effective in pro-

tecting against IAA. A surprising fact to emerge was the precipitous disappearance of the azide effect at high concentrations (0.25 M and above) of the azide. It may be pointed out that this result is not easily explained on the basis of an interaction between the IAA and NaN_3.

A comparison was also made of various concentrations of NaN_3 at several different concentrations of IAA. It was found that, if the concentration of the latter equaled or exceeded 1×10^{-3} M at pH 4.5, no protective effect with azide could be obtained, regardless of the concentration of the latter.

To ascertain whether this phenomenon was unique to IAA the effect of NaN_3 on other inhibitors with similar properties were examined. Three others were examined, namely, p-chloromercuribenzoate, phenyl

TABLE III

Per cent Inhibition Achieved in 20 Mins. by Various Glycolytic Poisons in the Presence and Absence of 0.01 M NaN_3

Agent	Concentration	Without azide	With azide
	M		
p-Chloromercuribenzoate	5×10^{-5} ·	32	8
	7×10^{-5}	40	19
Phenylmercuric acetate	1×10^{-5}	69	30
	2.5×10^{-5}	84	60

mercuric acetate, and iodoacetamide. The first two agents are extremely effective inhibitors of fermentation, so that it was necessary to use low concentrations to observe the phenomenon. The concentration range within which decreased sensitivity was observed in the presence of NaN_3 was relatively narrow as compared with iodoacetic acid. Table III summarizes some experiments testing the effect of 0.01 M NaN_3 on the ability of these two glycolytic poisons to inhibit fermentation of yeast suspended in $M/15$ KH_2PO_4 buffer. The experiments with p-chloromercuribenzoate were carried out at pH 5.6, whereas the others were performed at pH 4.5.

As in the case with IAA, at high concentrations is became impossible with any of these agents to detect the azide protective action because of the rapidity with which complete inhibition set in. Nevertheless, as is evident from the results recorded in Table III, it was possible in the case of each of these glycolytic poisons to find a concentration at which

the phenomenon of decreased sensitivity in the presence of azide could be exhibited.

It is interesting to note that this could not be done in the case of iodoacetamide. At no concentration of this latter agent could the protective action of NaN_3 be demonstrated. It is not obvious why the iodoacetamide should differ in this respect from the other inhibitors tested. It may, however, be remarked that, in agreement with previous investigators (23), much higher concentrations of the amide were required for effective inhibition of fermentation in yeast. Thus, to obtain 50% inhibition within 50 minutes, 1.25×10^{-3} M iodoacetamide had to be employed. This should be compared with complete inhibition obtained within 10 minutes for 2.5×10^{-4} M iodoacetic acid. Stannard (24) found a similar effect in muscle and further offered evidence (25) that the mode of action of IAA and iodoacetamide differed.

d. The Effect of NaN_3 on the Inhibition of Glycolysis with Sodium Fluoride

Warburg and Christian (26) have shown that the enzyme enolase which converts 2-phosphoglyceric acid to phosphopyruvic acid is a magnesium-protein complex which can easily be inhibited with sodium fluoride. In view of the above interpretation as to the locus of action of the NaN_3, experiments were performed to test the effect of azide on the inhibitory capacity of fluoride. Table IV summarizes the data obtained.

TABLE IV

The Effect of Azide on the Ability of NaF to Inhibit Fermentation

The numbers represent the percentage inhibition of fermentation attained 20 mins. after addition of fluoride in the presence and absence of 0.005 M NaN_3.

Molar concentration of NaF	Without azide	With azide
0.01	100	100
0.005	100	100
0.004	47	94
0.003	0	65
0.002	0	37
0.001	0	0

As in previous experiments, the tests were made with yeast cells suspended in $M/15$ KH_2PO_4 at pH 4.5. The fermentation was allowed to proceed for about 50 mins. previous to the fluoride addition, by which time an accurate determination of the rate was obtained. Following this period the proper amount of fluoride was introduced from the sidearm. It was found that, at the end of 20 mins., the full inhibitory power of the fluoride was exerted and the fermentation rate became linear at a rate

characteristic of the fluoride concentration employed. Consequently, the rate attained 20 mins. subsequent to the addition of fluoride was used to measure the degree of inhibition.

Examination of Table IV reveals that the response of the fermentation to fluoride in combination with azide is quite different from that observed with those glycolytic poisons which presumably act at the dehydrogenase level. The sensitivity to fluoride poisoning, instead of being decreased in the presence of NaN_3, is actually considerably increased. Thus, concentrations of NaF (0.003 M and 0.002 M) which have no detectable effect on the fermentation in the absence of azide cause considerable inhibition when this agent is present.

e. Some Alternative Interpretations of the Azide Effects

The experiments described in the previous sections have exhibited the following two facts.

(1) Azide dissociates the coupling mechanism which normally exists between oxidation and the esterification of inorganic phosphate.

(2) The presence of azide renders fermentation relatively resistant to poisons which can inhibit the enzyme controlling the coupling mechanism.

It seems unlikely that these two facts are unconnected and result from independent activities of the NaN_3. There are two hypotheses one might offer to explain the results thus far described. They may most easily be discussed with the aid of Fig. 4 which describes the pertinent steps of the glycolytic mechanism and the enzymes involved.

The first possibility (Path A of Fig. 4) supposes that the presence of NaN_3 makes it possible for the 3-phosphoglyceraldehyde to be oxidized directly without going through the steps mediated by the triosephosphate dehydrogenase. This would explain both of the facts mentioned, since no phosphate esterification would then occur and, furthermore, the poisoning of the dehydrogenase would not, under these conditions, lead to inhibition of fermentation. The second hypothesis (Path B of Fig. 4) assumes that the presence of NaN_3 permits the direct and immediate conversion of 1,3-diphosphoglyceric acid to 3-phosphoglyceric acid, thus avoiding the necessity of transferring the phosphate in the one position to ADP *via* the phosphopherase. The net result would be

that no phosphate esterification would be observed because, as soon as the organic phosphate bond is formed, it would be split. The relative insensitivity to poisons of the dehydrogenase can also be understood in terms of this mechanism if, as is often assumed to be the case,
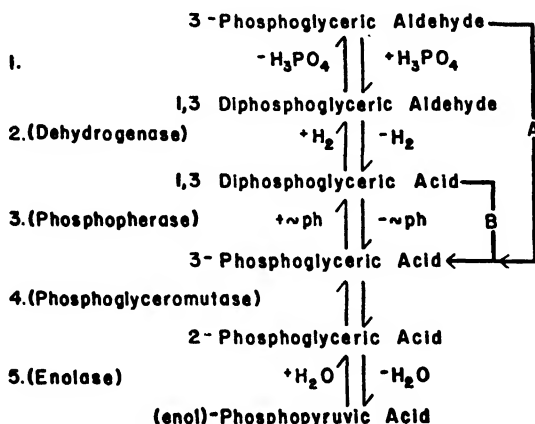
FIG. 4. The two alternative bypasses, A and B, within the glycolytic mechanism which could account for the effects of NaN_3 on the properties of fermentation and phosphate metabolism. See text for further discussion.

step 3 is the rate-limiting reaction of the glycolytic mechanism and is much slower than the dehydrogenase step. Since, in the presence of azide, a large fraction of phosphopherase is by-passed, the products of the dehydrogenase activity are much more quickly removed, thus permitting an apparent increase in the activity attained per enzyme molecule. Under such conditions, only a small fraction of the initial number of enzyme molecules present need remain functional to maintain the activity characterized by the normal fermentation. One may understand in this way why the addition of the IAA in the presence of azide leads to a relatively slow reduction of activity as measured by the overall fermentation rate.

There are several objections which may be raised against the first hypothesis involving Path A of Fig. 4. First, there is tacitly assumed the existence of another pathway of anaerobic oxidation for which no direct evidence exists. If it does exist, it is difficult to see why its activity should not be evoked by any poison (*e.g.*, IAA) which blocks the classical pathway at the dehydrogenase level, and why NaN_3 is nec-

essary for its activation. It is also not readily apparent why these same glycolytic poisons eventually do inhibit the fermentation in the presence of azide.

f. The Effect of Arsenate on the Inhibition of Glycolysis

The observations of Needham and Pillai (27) suggested a method for investigating the relative applicability of the two hypotheses outlined above. These authors noted that the oxidation of the triosephosphate was not coupled with esterification of inorganic phosphate in the presence of Na arsenate. This phenomenon was elucidated by Warburg and Christian (28) who assumed that arsenate, like phosphate, formed an intermediate compound with phosphoglyceraldehyde but, unlike 1,3-diphosphoglyceric acid, the oxidation product, 1-arseno-3-phosphoglyceric acid, decomposed very rapidly into the 3-phosphoglyceric acid and arsenate.

The functioning of this mechanism would tend to make arsenate-poisoned fermentation independent of phosphopherase, and a situation would obtain which would be equivalent to, but not necessarily identical with, Path B of Fig. 4. In any event, the conditions described as explanatory of the relative insensitivity of azide-poisoned fermentation to IAA inhibition would apply equally well to arsenate-treated cells. If, therefore, Path B were a correct formulation, it would follow that arsenate, like azide, would exhibit protective action against IAA.

Experiments to test this prediction were carried out in a manner exactly similar to those already described for NaN$_3$ with the exception that 24-hour cultures were used. The arsenate was put in the main compartment with the yeast. After establishing anaerobiosis and equilibration, the glucose (sufficient to make a final concentration of 3%) was introduced from one sidearm. After a steady rate of fermentation was established and measured, the IAA was tipped in from the other sidearm.

TABLE V

The Effect of Arsenate on the Ability of Monoiodoacetic Acid (IAA) to Inhibit Fermentation

The numbers represent percentage inhibition of fermentation attained 30 mins. after the addition of the IAA in the presence and absence of 0.01 M Na$_2$HAsO$_4$.

Experiment no.	Without arsenate	With arsenate
1	95	30
	95	31
2	98	21
	99	20

Table V records some typical results obtained at one of the arsenate concentrations tested. The numbers represent the percentage of inhibition attained 30 mins. subsequent to the addition of the IAA.

It was found that arsenate conferred "protection" against inhibition of fermentation by IAA. All of the other properties of the aresenate-poisoned fermentation examined were found to be similar to those observed in azide-treated cells. The parallelism extended even to increasing sensitivity to fluoride. The results of such experiments are summarized in Table VI. They were performed in a manner similar

TABLE VI

The Effect of Na_2HAsO_4 on the Ability of NaF to Inhibit Fermentation

The numbers represent the percentage inhibition of fermentation attained 20 mins. after the addition of the NaF in the presence and absence of 0.005 M Na_2HAsO_4.

Molar concentration of Na_2HAsO_4	Molar concentration of NaF	Degree of inhibition
0	0.003	0
0.02	0.003	42
0	0.002	0
0.02	0.002	35
0.01	0.003	18
0.01	0.002	0

to those already described in the azide case. Fermentation of 3% glucose was allowed to proceed anaerobically in the presence and absence of arsenate until a steady rate was attained. Appropriate amounts of fluoride were then introduced from the sidearms. The rates became stabilized 20 mins. subsequent to the fluoride addition and the degree of inhibition could be measured. As may be seen from the figures of Table VI, concentrations of fluoride, which, in the absence of arsenate, have no detectable effect on the fermentation, exert considerable inhibition in the presence of 0.02 M Na_2HAsO_4. At the lower concentration (0.01 M) of arsenate employed only the 0.003 M NaF exhibited any capacity to inhibit.

The experiments with arsenate indicate that an agent which can prevent the accumulation of diphosphoglycerate modifies the fermentation so that it becomes relatively insensitive to glycolytic poisons which can act at the triosephosphate dehydrogenase and becomes much more sensitive to inhibition by fluoride. In view of the great similarity between the effects of azide and arsenate on the fermentative system, it seems reasonable to conclude that they are acting at more or less the same enzymatic level with equivalent results. This would, therefore, accord with the hypothesis of azide action which is pictured by Path B of Fig. 4.

It is apparent from an examination of Pathway B that it can be realized by several quite distinct chemical mechanisms. We may summarize some of the possibilities in the following manner:

(1) Azide may act in a manner exactly equivalent to arsenate by replacing phosphate in the coupled oxidation to form the acyl azide. This compound, like its arsenate analogue would be highly unstable.

(2) The coupled oxidation may occur normally but the azide may interact with the product in such a way as to catalyze the splitting of the acyl phosphate.[5] This replacement reaction in turn could occur in any one of the following ways:

   a. The reaction may occur between azide and the acyl phosphate of free diphosphoglycerate.

   b. The reaction may occur between azide and the acyl phosphate, while the diphosphoglycerate is combined with the dehydrogenase.

   c. The reaction may occur while the diphosphoglycerate is combined with the phosphopherase.

Subsequent sections describe experiments designed to see which of the above possibilities is the most likely.

g. Azide and Acetyl Phosphate

The simplest possibility of a direct replacement reaction between azide and acyl phosphate was considered. An examination was made of the effect of NaN_3 on the spontaneous decomposition of synthetic acetyl phosphate[6] dissolved in an aqueous medium. Solutions containing 6 micromols/cc. were prepared according to Lipmann and Tuttle (30) in phthalate buffer adjusted to various pH values, including the one of maximum stability. The spontaneous hydrolysis of the acyl phosphate in the presence and absence of azide was compared. The extent of the hydrolysis was assayed by acyl phosphate determination

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[5] Model reactions of this type involving acyl chloride and azide are often used in the preparation of acyl azides. In this instance an acyl chloride is allowed to stand in a non-aqueous medium with dry NaN_3. As a result, the chloride is split off by replacement with azide to form acyl azide. The resulting compound is highly unstable and can be decomposed to form the isocyanate with the evolution of nitrogen, a reaction termed the Curtius rearrangement (29).

[6] Di-Ag-acetyl phosphate was obtained through the kindness of Dr. D. Rittenberg.

with the hydroxylamine reagent (31). In no case, at any of the pH values tried, could any effect of NaN₃ on the hydrolysis be detected, although concentrations as high as 0.2 M NaN₃ were included.

h. The Effect of NaN₃ on the Triosephosphate Dehydrogenase System[7]

The analysis of several of the other possibilities raised was undertaken with the aid of the isolated triosephosphate dehydrogenase. The reaction system contained crystalline triosephosphate dehydrogenase, glyceraldehyde phosphate, and diphosphopyridine nucleotide. Sodium pyrophosphate adjusted to pH 7.4 was used as the buffer system. The substrate was obtained as the dioxane compound, and was prepared by dissolving the appropriate amount and carefully bringing the solution to neutrality. All reagents which were added were dissolved in a solution which was 0.03 M in sodium pyrophosphate and 0.06 M in cysteine hydrochloride and adjusted to pH 7.4 when necessary. All reagents were freshly prepared immediately before use. Activity was ascertained by measuring extinction coefficients at λ 340 mμ with a Beckman spectrophotometer. Further details concerning composition of the reaction mixtures will be found by consulting the tables and graphs of the experiments cited.

Table VII gives the results obtained in experiments designed to test the possibility that azide can, like arsenate, replace phosphate in the coupled oxidative reaction. A comparison was made of activity observed in reaction mixtures in which arsenate and azide were present alone and in combination. Col. 1 gives the activity observed with arsenate. Within 6 mins. almost complete reduction of the added coenzyme is attained. As may be seen from Cols. 2 and 3, neither 0.002 M nor 0.03 M NaN₃ enables the reaction to proceed. A comparison of Col. 1 and 4 shows that the presence of NaN₃, even in as high a concentration as 0.03 M, has no detectable effect on the progress of the reaction when arsenate is participating.

It is clear from these results that azide cannot replace phosphate or arsenate in the reaction and, furthermore; that it has no detectable effect on the activity of the enzyme.

The possibility of a replacement reaction between the azide and the acyl phosphate of the diphosphoglycerate, either free or combined with

TABLE VII

A Comparison of NaN₃ and Na₂HAsO₄ in the Functioning of the
Triosephosphate Dehydrogenase System

The reaction mixtures contained in addition to the components noted below, the following: diphosphopyridine nucleotide (0.00027 M); cysteine hydrochloride (0.06. M); sodium pyrophosphate (0.03 M); glyceraldehyde phosphate (0.005 M); triosephosphate dehydrogenase 4 γ. The total volume in all cases was 3 cc. and the pH was adjusted to 7.4.

Reagent	Concentration	1	2	3	4
		cc. Added			
	M				
Na₂HAsO₄	0.3	0.1	0	0	0.1
NaN₃	0.02	0	0.3	0	0
NaN₃	0.3	0	0	0.3	0.3
Time		Readings at λ 340 in extinction coefficients			
mins.					
0.5		0.60	0.05	0.05	0.58
1.0		0.95	0.06	0.05	0.95
2.0		1.35	0.06	0.05	1.33
3.0		1.64	0.06	0.05	1.62
4.0		1.82	0.06	0.06	1.78
6.0		1.92	0.05	0.06	1.84
8.0		1.95	0.06	0.06	1.93
10.0		1.97	0.06	0.06	1.96

the dehydrogenase, was next examined. This was done in experiments in which the systems employed were identical to those described in Table VII with the exception, however, that orthophosphate rather than arsenate was present. To detect the presence of a replacement reaction, the amount of orthophosphate added was made limiting, so that only a fraction of the added coenzyme would be reduced at the equilibrium position assumed. The occurrence of any replacement reaction would liberate orthophosphate and raise the equilibrium position toward complete reduction of the coenzyme. Fig. 5 gives the results of some experiments of this nature. In these experiments the effects of 0.02 M azide at two different orthophosphate concentrations were examined. The points lying along Curve I give the observations on reaction mixtures which were 0.001 M with respect to phosphate and in which about 12% of the added coenzyme is reduced at the

equilibrium position. It is evident that azide has no effect, since the points for the mixture containing azide fall on the same curve as the one without azide. The second group of curves (II) illustrate the same type of experiment performed at 0.03 M phosphate. The latter concentration, under the conditions of these experiments, permits reduction of 50% of the added coenzyme. Here again it is clear that no effects of azide were observed. At the time indicated by the arrow,
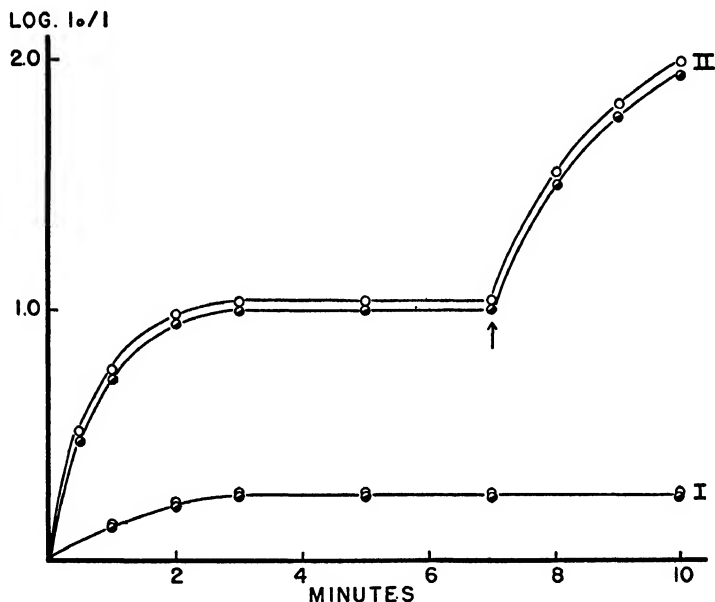
FIG. 5. Effect of azide on the triosephosphate dehydrogenase system with limiting amounts of phosphate. Open circles refer to controls and shaded circles to mixtures containing NaN₃. The lower curves (I) represent experiments run in the presence of 0.001 M phosphate. The upper curves (II) are similar experiments in the presence of 0.033 M phosphate. The arrow indicates the time of addition of arsenate.

arsenate was added to the two mixtures and the subsequent response proves that both the experimental and control systems were fully functional.

In addition to the experiments reported, a large number of attempts were made to vary the conditions in directions which might conceivably magnify the existence of any possible interactions with azide. These attempts included preincubation of enzyme and various com-

ponents of the system with azide in a manner analogous to the intact cell experiment. Further, in some experiments the concentrations of the reactants were increased by a factor of 20 in an effort to take advantage of any mass action effects. In none of these was any positive evidence obtained for an interaction between azide and any component of the triosephosphate dehydrogenase system, either alone or in combination.

DISCUSSION

a. A Possible Mechanism of Azide Action

All the facts ascertained in these researches on the properties of azide-poisoned fermentation by intact cells indicate the capacity of this agent to nullify the effects of the coupled oxidative step. The inability to esterify phosphate, the concomitant loss of synthetic capacities, the insensitivity of the fermentation to such agents as iodoacetic acid, and finally, the complete parallelism with arsenate-poisoned fermentation, all point in the same direction. Several of the detailed chemical mechanisms which were offered to explain the phenomena observed in the presence of azide have either been completely eliminated or made unlikely by the experiments with the isolated dehydrogenase system.

The evidence is clear that azide does not, like arsenate, enter directly into the oxidative reaction and thereby interfere with acyl phosphate formation. Its action would appear to be effective only after the oxidation has taken place. It is equally evident that the presence of azide results in no detectable hindrance to the functioning of the enzyme when either arsenate or phosphate is participating in the reaction.

With respect to the replacement reaction hypothesis however, the evidence is as yet not unequivocal. Within the confines of this suggestion, certain particular mechanisms have been made highly unlikely. Thus, in view of the completely negative results with the isolated system, it does not seem probable that azide can replace the acyl phosphate of free diphosphoglycerate. On the same basis it is not probable that the reaction occurs while the product is still combined with the triosephosphate dehydrogenase. The most likely possibility, therefore, is that azide can split off the acyl phosphate when the diphosphoglycerate combines with the phosphopherase.

The following may then be proposed as an explanation of the properties of azide-poisoned fermentation, where E is the enzyme phosphopherase and ph refers to $H_2PO_4^-$.

$$ph\text{-glyceryl} \sim ph \cdot E + N_3^- \rightarrow ph\text{-glyceryl} \sim N_3 + E + ph, \quad (1)$$

$$ph\text{-glyceryl} \sim N_3 + OH^- \rightarrow ph\text{-glycerate} + N_3^-. \quad (2)$$
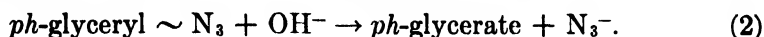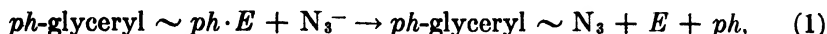
In these equations Lipmann's (1941) convention $(\sim ph)$ of an energy-rich phosphate bond is employed. This mechanism would aid in understanding the considerable efficiency of azide as an inhibitor of both P uptake and turnover. It would tend to insure that each recently esterified orthophosphate would be particularly prone to cleavage as soon as it became attached to the phosphopherase for transfer into the common pool of organic phosphate via ATP.

By the very nature of the enzyme concerned, and its function, it would seem necessary that the attachment between the diphosphoglycerate and the phosphopherase involves the acyl phosphate. A mechanism is thus provided whereby azide could increase the rate of turnover by the enzyme since the replacement reaction accomplishes not only the mineralization of the organic phosphate but also the simultaneous removal of the product from the enzyme surface.

b. The Kinetics of IAA Inhibition in the Presence of Azide

Effectively, a shunt across the phosphopherase is provided, which releases the glycolytic cycle from the regulatory restraints of limited phosphate acceptors. Under these conditions, the reaction rate would no longer be determined by the level of adenosinediphosphate available for removal of the acyl phosphate of diphosphoglycerate. This provides an explanation for the time course of the azide protection against IAA inhibition. The rate of fermentation is ordinarily limited by the accumulation of the products of triosephosphate dehydrogenase activity. In the presence of IAA the effective enzyme concentration is lowered, but this is compensated by the rapid removal of the diphosphoglycerate. As time goes on, less and less functional enzyme is available and, because the azide can only react with the product formed by the enzyme, the acceleration in the reaction due to splitting of the acyl phosphate no longer compensates for the lowered concentration of enzyme. Eventually, of course, the fermentation ceases completely.

c. Increased Sensitivity to Fluoride in the Presence of Azide and Arsenate

The mechanism underlying the increased sensitivity to fluoride in the presence of both azide and arsenate is not so easily specified. As pointed out previously, both of these agents would effectively permit the fermentation to bypass the phosphopherase and, therefore, to pile up at the enolase level. Either this, or some following fluoride-sensitive reaction, could then become rate-limiting, resulting in an increased sensitivity to poisoning by fluoride. Another possible explanation may lie in the problem of maintaining an adequate ATP level in azide- or arsenate-poisoned cells. In the presence of either of these two agents, little, if any, ATP is generated as a result of the coupled oxidation. The regeneration of ATP under these circumstances can come only from the transfer from phosphoenolpyruvate to adenosinediphosphate. If this reaction is rendered less efficient by the presence of fluoride, a fall in the available ATP would occur which could not be compensated. A lower fermentation rate might then result. In this connection, it is of some interest to recall a peculiar property of fermentation inhibition by fluoride. With such agents as iodoacetic acid, as soon as inhibitory concentration ranges are reached, one finds that the introduction of the agent leads ultimately to complete suppression of the fermentation. The speed with which the total abolition of activity is attained varies with the concentration of the agent employed and the conditions under which it is acting. In the case of fluoride, however, what occurs is a suppression to a lower level of activity, which is then maintained more or less indefinitely. It is as though what were occurring was not progressive destruction of enzyme but an interference with the efficiency with which it operated.

d. The Dissociation of Glycolysis from Synthetic Activity

Any attempt to explain the capacity of azide to uncouple glycolysis from the utilization of the energy generated by this process requires an adequate interpretation of two facts. One is the loss of synthetic capacity and the other is the apparent maintenance of the glycolytic cycle in full functional activity.

If the generally accepted assumption that phosphate bonds form the primary energy source for synthetic activity is adopted, then the ability of azide to inhibit synthesis of various kinds is adequately ex-

plained by the findings reported in parts A and B of the experimental section. These experiments show that azide interferes with the formation of organic phosphate bonds. Independently of the details whereby this is accomplished, it would be expected from these results alone that little or no synthetic activity would be found in azide-poisoned cells. To explain the second fact mentioned, however, it is evident that this interference with phosphate esterification must be such as to permit the glycolysis to continue. This condition receives adequate explanation in terms of the mechanism proposed for uncoupling the generation of phosphate bonds from the glycolytic cycle.

Two ATP molecules are required per glucose molecule fermented to maintain the glycolytic system in operation. Normally, four are produced per molecule, two as a result of the coupled oxidation and two from the production of phosphoenolpyruvate. Thus, for every molecule of glucose fermented by the normally functioning system, two extra ATP molecules are produced, which can be used for synthetic purposes. In the presence of azide, according to the suggested mechanism, the two normally formed as a result of the phosphopherase activity are not realized. Thus, under these conditions only two ATP molecules are generated for every glucose molecule glycolyzed. Sufficient ATP is, therefore, supplied for the maintenance of glycolysis but no extra ones are formed to be employed in energy-utilizing synthetic reactions.

The hypothesis, therefore, explains both aspects of the uncoupling mechanism. One may also understand the wide variety of synthetic activities inhibited by azide. This property is, from the point of view of the proposed mode of action, primarily attributable to interference with one of the basic energy sources of anaerobic metabolism.

e. Conclusion

The evidence leading up to the proposed mechanism for azide action is as yet admittedly indirect. It is based on the analysis of the azide-poisoned fermentation of intact cells and the elimination of the immediately testable alternatives with *in vitro* experiments. Relatively conclusive data on the existence of a replacement reaction between azide and the acyl phosphate of the diphosphoglycerate-phosphopherase complex may be obtained by experiments with the isolated enzyme system. Such experiments represent the next obvious step in the investigation of the mode of action of azide.

To assay the weight to be placed on the conclusions drawn from the present experiments, it is of some value to note specifically the tacit assumptions which are generally made in formulating hypotheses such as the one proposed here. One such assumption is that it is possible to. transfer results obtained with isolated enzyme systems to the intact cells. It is on this basis that the alternative explanations involving the components of the triosephosphate dehydrogenase system were tentatively eliminated. Another assumption made is that our knowledge of anaerobic glycolysis is sufficiently accurate and complete to justify specifying the coupled oxidative step as the major one concerned with the formation of organic phosphate bonds during the anaerobic metabolism of carbohydrate. If, in particular, this last assumption is correct, then it is difficult to avoid implicating either the oxidative step or the product of its activity as a component of the mechanism whereby azide exerts its effect. If these can be shown not to be involved, it will be necessary to suppose that the assumption of the completeness of our knowledge on this point is incorrect. In that case, one would have to examine the possibilities that there exists an as yet unidentified component of the dehydrogenase system which was not included in the *in vitro* tests, or that an unknown but quantitatively important mechanism for phosphate esterification functions in the intact cell which is sensitive to azide.

At present, the replacement reaction mechanism at the phosphopherase seems to be the simplest hypothesis which will explain the already diverse set of facts available on the chemical and biochemical properties of azide.

In conclusion, a few remarks may be appended concerning results obtained in an investigation of these phenomena in Lebedev preparations. These results will be reported in detail in a subsequent paper, since they raise problems which are not pertinent to the subject matter of the present paper. However; it may be noted that azide, at concentrations completely effective in preventing phosphate esterification in the intact cell (5×10^{-3} M), is not capable of inhibiting this process in the cell-free extract. This loss of inhibitory capacity by azide in extract is not limited to yeast or to the phosphate-esterifying enzymes. An analogous situation has been reported (32) with respect to the ability of this agent to inhibit the cytochrome oxidase system. Concentrations of sodium azide which are extremely effective in suppressing the respiration of intact frog .embryos have no effect on respiring

extracts prepared from them. That this situation is not due to a change in the sensitivity of the enzyme is indicated by the fact that sodium cyanide is equally effective as an inhibitor of the respiratory mechanism in both situations. On the basis of preliminary experiments, it is likely that the excessive amount of dissolved protein found in such cell-free preparations interferes by binding the azide. Interactions between protein and azide have been observed (33, 34). Whatever the reason for this loss of inhibitory ability by azide in cell-free extracts, its existence complicates attempts to compare *in vivo* and *in vitro* behavior of enzyme systems with respect to this agent.

SUMMARY

An experimental analysis of the known ability of NaN_3 to dissociate anaerobic glycolysis from cellular synthetic activity is presented. The following pertinent findings may be noted:

1. The ability of the cell to esterify inorganic phosphate was greatly suppressed at azide concentrations which did not interfere with the metabolism of glucose. The exchange of internal and external phosphate as measured with P^{32} was also inhibited by the presence of azide.

2. Azide decreased the sensitivity of the fermentation to poisons which inhibit the triosephosphate dehydrogenase.

?. Sensitivity of the fermentation to fluoride was increased in the presence of azide.

4. The response of arsenate-poisoned fermentation to dehydrogenase inhibitors and sodium fluoride was found to be similar to that observed in the case of azide-treated cells.

5. The possibility that azide can, like arsenate, replace phosphate in the coupled oxidation was eliminated by *in vitro* experiments with the triosephosphate dehydrogenase. Further, no effects of azide on the functioning of this enzyme were observed when either phosphate or arsenate was participating.

On the basis of these findings it is suggested that azide uncouples anaerobic oxidation from synthesis by means of a replacement reaction between azide and the acyl phosphate of diphosphoglycerate while the latter is combined with phosphopherase. The resulting acyl azide,

being unstable, would hydrolyze rapidly. The properties of azide-poisoned fermentation noted above are interpreted in terms of this mechanism.

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The Interrelations of Choline and Glycine Betaine in the Growth of the Chick

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INTRODUCTION

This paper deals with the quantitative relations of choline and glycine betaine to growth, and with the extent to which betaine can replace choline. Earlier work has shown that a mixture of betaine and arsenocholine produces as much growth as choline, but that with either alone growth is less (1). Since arsenocholine is found in the tissues (2), but does not appear able to act as a transmethylating agent (3), and since betaine has only been found in mammalian tissues in very small traces (4), it has been presumed that betaine assumes the transmethylating role of choline and that arsenocholine replaces it as a tissue constituent.

EXPERIMENTAL METHOD

The chicks used were single-comb White Leghorns. The first 3 weeks of life constituted the conditioning period, during which the birds were fed a diet sufficiently low in choline to produce incipient perosis. The fourth week was the test period. The increase in weight from the 14th to the 20th day was measured and 10 birds selected for each experiment such that they formed a series showing both increasing weight gain during this period and increasing final weight at the end of the period. Each of the 10 birds in a pen, then, had companions in all the other pens showing close to the same growth during the last week of the pretest period and close to the same weight at the end of the period. In selecting birds, all those showing very large or small weights were discarded, as well as those showing large or small weight gains compared to their weight at the end of the pretest period. The birds were put on the test diet on the 21st day and weighed daily until the 28th day. The growth was determined by plotting the weights against time from the 22nd to the 28th day, drawing the best straight line, determined by inspection, through the points, and calculating from this line the increase in weight per day, as a percentage of the average weight during the period. "In drawing this line, it is kept in mind that what is wanted is a measure of the

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normal reaction to metabolic causes uncomplicated by chance variation. For example, if a bird fails to eat on one day, and so a single point falls very much below the trend established by the other points, it is disregarded in drawing the line" (5).

Examination of about 200 birds from different hatches and fed different diets has shown that growth during the fourth week of life varies inversely with the weight at the beginning of the period. The regression coefficient is close to 0.0175% decrease in growth/g. increase in initial weight. It is evident then, that, though it is necessary to use birds of different initial weight, greater accuracy in comparison can be obtained by adjusting the growth of all lots to that for a standard initial weight, using the above regression coefficient. In making such adjustments the growth of each bird in a pen has been plotted against its initial weight and a line with the slope of the above regression coefficient drawn through the mean value. Occasional growths that depart more than 1% from this line have been discarded (5). With the degree of selection and number of chicks used, the average growths are reproducible to about 0.1%. Note for example, in Table I, the agreement in the adjusted growths of pens 14 and 15. A statistical examination of this method of selecting birds and evaluating growth has been presented elsewhere (5).

DIETS

The Low Choline Diet contained (in per cent): gelatin 8; casein 18; glucose 45; yeast (Anheuser-Busch strain G yeast—choline content 0.36) 9; and basal supplements: $Ca_3(PO_4)_2$—2.000, NaCl—1.000, $MgSO_4 \cdot 7H_2O$—0.560, K_2HPO_4—0.500, KCl—0.300, $Na_2SiO_3 \cdot 6H_2O$—0.200, $Al_2(SO_4)_3 \cdot 18H_2O$—0.100, KI—0.001, FeCitrate—0.074, $MnSO_4 \cdot H_2O$—0.030, $CuSO_4$—0.003, $ZnSO_4$—0.005, $Ca(C_2H_3O_2)_2 \cdot 4M_2O$—0.002; 2-Methyl-1,4-naphthohydroquinone diacetate—0.001, inositol—0.001, cholic acid—0.100, biotin—0.00001, CaGluconate—8.000, vitamin E conc. (15% mixed tocopherols)—0.03, sardine oil (400 A.O.A.C. units vitamin D and 3000 I. U. vitamin A/g.)—0.25, cottonseed oil—5.30, cellulose—6.0.

The Basal Diet During the Test Period contained (in per cent): Isolated soybean protein—23 (containing 87 protein and equivalent to 0.30 methionine and 0.05 cystine); glucose—45; thiamine—0.001, riboflavin—0.001, pyridoxine—0.001, CaPantothenate (*dl*)—0.004, nicotinic acid—0.003; and other basal supplements as above. The choline content of this basal diet was inappreciable.

The Supplements are shown in Table I, Cols. 2 and 3. The methionine content of the diets, 0.30%, was considered sufficient when combined with adequate cystine (0.05% basal plus 0.50% supplement) to provide most, but not all, of the requirements for methionine as a tissue constituent, but insufficient to provide transmethylating capacity, assuming these two functions to be mutually exclusive. Combined with the 0.23% homocystine, the cystine and methionine were considered adequate to provide all the requirements for sulfur amino acids (6).

RESULTS

Table I shows (Cols. 4 and 5) the initial weights and actual growths, as pen average, and the growths (Col. 6) adjusted to the standard initial weight of 100 g.; the latter values only will be used in subse-

TABLE I

Dietary Supplements and Chick Weights and Growth[a]

1	2	3	4	5	6
	Supplements[b]		Initial weight	Growth	
Pen no.	Choline Cl	Betaine HCl		Experimental	Adjusted to initial wt. 100 g.[c]
	Parts added to 100 parts basal diet		*g.*	*Per cent*	*Per cent*
1	0.0273	0.01	105.5	2.00	2.10
2	0.0820	0.01	104.0	3.91	3.98
3	0.0455	0.05	106.1	3.52	3.63
4	0.0273	0.07	104.5	3.46	2.54
5	0.1730	0.01	124.1	5.36	5.78
6	0.1360	0.05	125.0	5.24	5.68
7	0.1160	0.07	124.0	5.22	5.64
8	0.0820	0.11	126.0	5.06	5.51
9	0.0455	0.15	124.2	4.78	5.20
10	0.0273	0.17	124.5	4.03	4.46
11	0.1730	0.07	106.1	5.97	6.08
12	0.1360	0.11	104.9	6.19	6.28
13	0.0820	0.17	107.0	6.18	6.30
14	0	0	109.8	0.84	1.01
15	0	0	131.3	0.48	1.03

[a] Hatching date Nov. 1, 1944.

[b] Each diet except 14 and 15 had 0.50 parts L(-)-cystine and 0.23 parts DL-homocystine added to 100 parts basal diet.

[c] Coefficient of regression 0.0175%/g. increase in initial weight.

quent discussion. In Fig. 4 the adjusted growths from certain other experiments covering a wider range of choline and betaine concentrations are shown (solid curves). These experiments were intended to supplement those recorded in Table I, but through an error only 4.5% of the strain G yeast was added to the low choline diet, so that these birds when put on the test diets were so highly perotic that, even after relaxing the criteria of selection, only 8 or 9 birds could be obtained for each experiment. The data are, therefore, not as precise as those in Table I, and, because of the different physiological condition of the birds, are not comparable. They are introduced, however, because they yield additional information.

DISCUSSION

In the following discussion it is assumed that absorption from the gut is essentially complete and that concentrations of ingredients in the diets are simply related to the concentrations of these ingredients available to the tissues. This is a simplification, since the amount of food eaten, and so of the supplement, must also be factors. But variation in food consumption is much less than variation in the concentra-
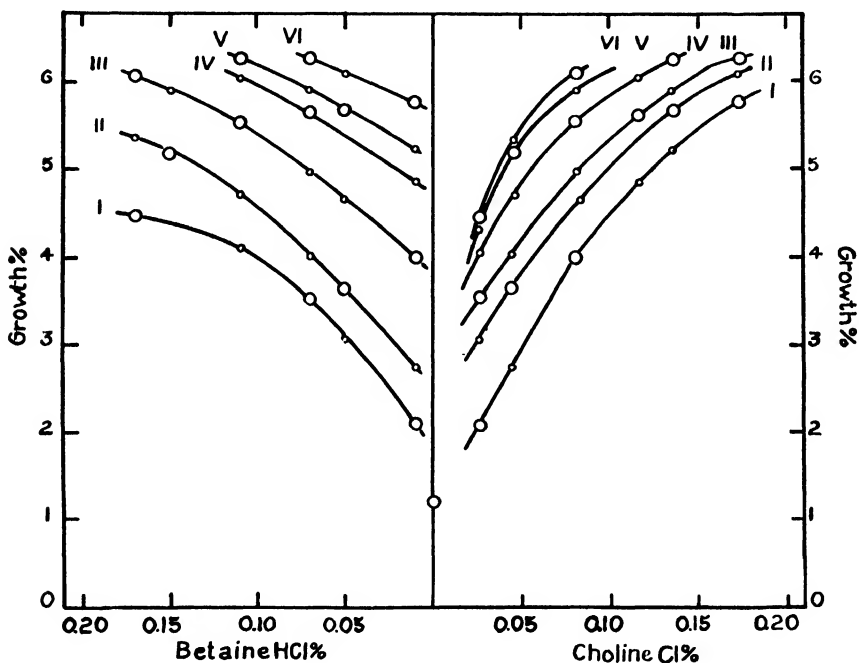
FIG. 1A. Relation of growth to concentration of glycine betaine HCl at constant concentrations of choline Cl. Curve Nos. and concentrations of choline Cl (in g./100 g. basal diet): I, 0.0273; II, 0.045; III, 0.082; IV, 0.117; V, 0.136; VI, 0.173.

FIG. 1B. Relation of growth to concentration of choline Cl at constant concentrations of betaine HCl. Curve Nos. and concentrations (in g./100 g. basal diet): I, 0.01; II, 0.05; III, 0.07; IV, 0.11; V, 0.15; VI, 0.17.

tion of the supplements—only about 10% as compared to several hundred per cent—a satisfactory analysis of results can be obtained by using the concentrations. This matter is more fully discussed and the above assumptions justified in another paper (10).

In Fig. 1 the growth is plotted against the concentrations of choline and betaine in the diets.

The two sets of curves are mutually consistent and it will be found that it is impossible to change any one of them without unreasonably distorting the corresponding curves in the other set. This means that the three-dimensional surface whose coordinates are choline Cl con-
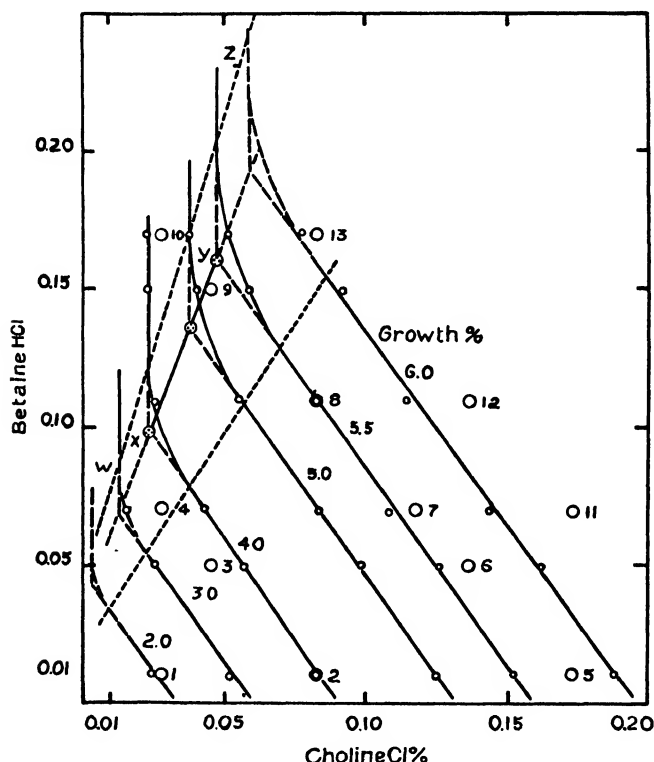
FIG. 2. Replacement of choline by betaine at several growths. Symbols: 0, exp. points; o, points cross-plotted from Fig. 1. (Note: This figure is analogous to a contour map in which growth replaces elevation above the datum plane and betaine HCl and choline Cl concentrations replace distances north and east.)

centration, betaine HCl concentration, and growth, is fairly well delimited by the 15 points. (The small circles represent points cross-plotted from one set to the other.) From these curves, Fig. 2 has been constructed by cross-plotting and shows the mutual requirements of choline and betaine to sustain growths of from 2 to 6%. (The small

circles represent the points obtained from Fig. 1, the large circles the experimental points.) It is unfortunate that the distribution of points is such that the curves are partly based on extrapolated portions of Fig. 1 (these parts are shown dotted). It must be remembered that conclusions based on these extrapolated parts are less well sustained than others and, in particular, that the quantitative relations found may be liable to some change. Nevertheless, it seems probable that the qualitative conclusions will not require much modification in the future.

The intersections of the lower ends of the curves with the abscissa give the choline required for each growth in the absence of betaine. The curves form in their lower parts a series of parallel straight lines indicating that the mol ratio at which betaine can replace choline remains constant during most of the course of the substitution and that this ratio is the same at all growths—1.42 on a weight basis, or 1.29 on a mol basis. This suggests that, allowing for some decrease in efficiency, betaine substitutes for choline mol for mol during most of the possible substitution.

The upper ends of the curves bend abruptly toward the vertical showing the limits to which betaine can substitute for choline. The vertical parts of the curves at growths 4.0, 5.0, and 5.5 are the best established and the values of the abscissae give the values of what may be called the essential choline at these growths. The intersection of the corresponding ordinates and the extrapolations of the straight diagonal parts of the curves gives points lying in a straight line XY (Fig. 2). Extrapolation of this line to intersect the extrapolated straight parts of the growth curves at other levels gives values for the choline essential for these growths falling within the limits indicated by the less well defined vertical parts of these curves, so it is probable (although not proved) that this straight line relation holds at all growths. The difference between the total choline and the essential choline at any growth is the choline replaceable by betaine.

The logs of these three fractions of the choline requirement are plotted against the growths in Fig. 3. Owing to the characteristics of Fig. 2 already mentioned, it should be noted that the curve for total choline is better established than the curves that show the essential and replaceable parts separately.

The curves for essential and replaceable choline at growths above 3% give straight lines which, in simple reaction kinetics, would indi-

cate first order reactions, if growth can be considered a measure of reaction velocity. And it might be expected that, when growth is limited by deficiency of only one dietary constituent, it would follow a pseudo-monomolecular course.

If the relation between essential and replaceable choline is plotted (curve not reproduced) a straight line is obtained which, however, does
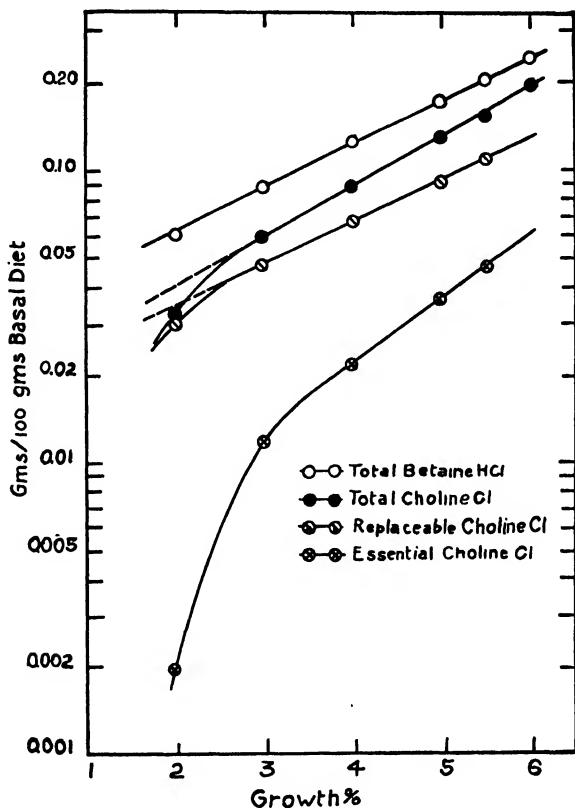
Fig. 3. Relation of growth to essential and replaceable choline and to betaine.

not pass through the origin, but shows zero essential and 0.26% replaceable choline required at a growth which, from Fig. 3, must be about 1%. The fact that some growth can be obtained without essential choline, provided replaceable choline or betaine is present, may be related either to the choline present in the tissues or to an ability to synthesize small amounts (this point is considered later). While

these birds had been fed a diet low in choline during the pretest period, it has not yet been shown that this decreases the tissue choline. The fact that the need for replaceable choline does not approach zero as growth approaches zero may be related to the fact that the diets con-* tained homocystine, which has been shown to be continuously methylated even in the presence of adequate methionine (7). Nevertheless, it should be noted that the homocystine fed (0.23%) was equivalent to 1.7 mM/100 g. of diet, while the replaceable choline required to sustain a growth of 6% is only 0.135% (1.0 mM) and to sustain a growth of 2% is 0.033% (0.2 mM). This may indicate that: (1) while the methylation of homocystine may go on continuously, it must go very incompletely at the lower growth levels; (2) part of the homocystine may be used up in other ways, such as the formation of cystine.

The curves in Fig. 2 approach the vertical asymptotically, making it impossible to read accurately the total amounts of betaine replacing choline. But a straight line (WZ) showing the approximate amounts has been drawn. If the values for betaine thus obtained are plotted against the replaceable choline, a straight line through the origin is obtained (curve not reproduced) the slope of which corresponds to 1.67 mols of betaine replacing one mol of choline. This ratio was 1.29 up to 80% replacement. The fact that this curve passes through the origin shows that the only function of betaine, under these conditions, is to replace part of the choline.

In relating these results to normal dietary needs, it must be remembered that the diets contained 0.23% homocystine, which may increase the need of remethylating agents. On the other hand, the birds had been fed a diet low in choline during the pretest period. And since it is probable that the initial growth response to a constituent in the diet that has previously been inadequate increases with the degree of the preceding inadequacy, choline levels required for a given growth might be somewhat greater in normal chicks.

It is uncertain how closely the quantitative relations developed above would hold for chicks of different age or fed different conditioning- or test-diets. Probably both the ratio of essential to replaceable choline and the need for total choline would vary with conditions, but it seems quite likely that under all conditions the replacement of choline by betaine would begin as a straight line relation which would be the same at all growths.

Some information on these points can be had from the results plotted in Fig. 4. The curves show the variation of growth with the choline content of the diet at several concentrations of betaine. The solid curves show the results obtained with birds drastically deprived of choline in the pretest period; the dotted curves are replotted from Fig. 1.
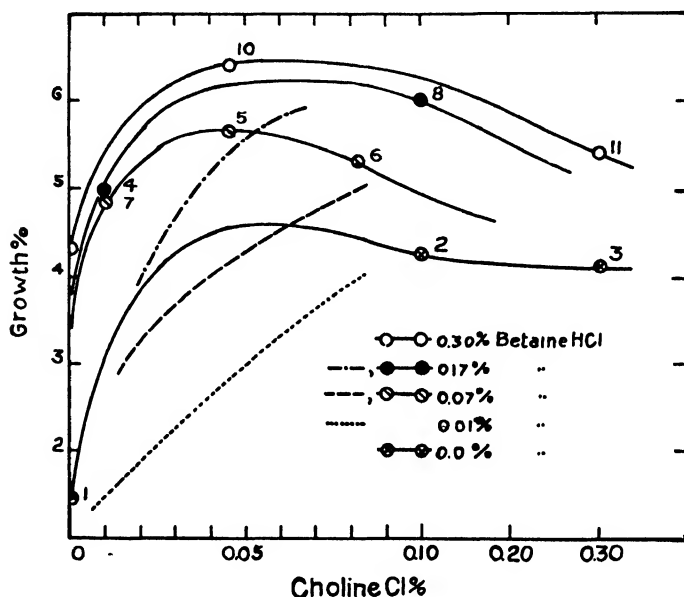
FIG. 4. Relation of growth to concentrations of choline and gylcine betaine in extremely perotic chicks. Concentrations in g./100 g. basal diet.

Comparison of the two sets of curves shows the much greater response to small amounts of choline of the birds that had previously suffered the greater deprivation. For example, in these highly perotic chicks 0.01% choline Cl plus 0.07% betaine HCl (Point 4) increased the growth to 4.8% from the basal value of 1.5% (Point 1). In the less deprived birds, the dotted curves show the same change to increased growth only to 2.7%.

Two other facts brought out by the solid curves should be noted. First, contrary to findings with the less deprived birds, betaine is sometimes, but not always, more effective in producing growth than an equivalent amount of choline. Comparison of Point 4 with Points 5

and 7, for example, shows that adding 0.10% betaine HCl to a diet already containing 0.01% choline Cl and 0.07% betaine HCl is ineffective in increasing growth, while adding 0.035% choline Cl increases growth from 5.0% to 5.6%. Growth here is probably limited by need for essential choline, which betaine cannot supply. Conversely, Point 2 (0.10% choline Cl—equivalent to 0.71 mM/100 g. diet) gives a growth of 4.2%, whereas Point 5, with nearly the same total molar concentration of supplements—0.045% (0.32 mM) choline Cl plus 0.07% (0.45 mM) betaine HCl, gives a growth of 5.6%, an increase of 1.4%. Since, in both cases, essential choline is probably being adequately supplied, the lack must be in replaceable choline, and so it appears that under certain conditions betaine is the more effective methylating agent. Since the 0.30% methionine in these diets is inadequate for maximum growth, it is probably the homocystine that is more effectively methylated by betaine.

A possible explanation can be offered: It has been shown recently that dimethylaminoethanol will support fair growth in the chick in the absence of choline (8). This may indicate the synthesis of small amounts of choline from dimethylaminoethanol and methionine with reduction of the latter to homocystine. But homocystine and choline will form methionine (1), so the equilibrium:

Homocystine + Choline $\leftrightarrows$ Methionine + Dimethylaminoethanol

may exist. This would reduce the effective concentration of methyionine. When choline is replaced by betaine the reaction may go only to the right. Considering the minute amounts of betaine that have been found in mammalian tissues (4) (no data are available for avian), its rapid demethylation to glycine (9), and the relatively large concentrations of methionine and glycine present, it seems unlikely that the reaction involving betaine would be reversible. It may be noted that best growth (6.4%) was obtained with 0.045% choline Cl and 0.30% betaine HCl, rather than with 0.30% choline Cl.

This last fact introduces the second point: the depressing effect of choline on growth when the concentration rises above about 0.10%, particularly at the higher levels of betaine. This effect will not be discussed. Later work has shown it to be related to the methionine supply and it has been considered in another paper (10).

In general, comparison of the solid and dotted curves in **Fig. 4** suggests that the greater inadequacy of the pretest diet causes a

squeezing together of the limits of the dietary ingredients required to produce a given response. Certainly the need of the highly perotic chick for essential choline is decreased, since a growth of 6.4% was produced by a diet containing only 0.045% choline Cl, all of which may not have been essential, whereas the birds in the better physiological condition required 0.06% essential choline Cl to produce a growth of 6.0%.

SUMMARY

(1) The conclusions that follow are based on experiments with incipiently perotic chicks, during the fourth week of life, using diets containing homocystine, and apply strictly only to such animals under such conditions.

(2) The choline required by chicks may be divided into 2 parts which may be called essential choline and replaceable choline. Essential choline is presumably used in tissue formation and replaceable choline in transmethylation. There is a straight line relation between the essential and the replaceable choline required as growth varies. Close to 0.06% essential choline Cl and 0.14% replaceable choline Cl is required to sustain a growth of 6%.

(3) Replaceable choline can be replaced by glycine betaine; essential choline cannot. The results indicate that the only function of betaine is to replace replaceable choline.

(4) In deficiency of choline or of choline plus betaine, the relation of growth to the concentration of the deficient ingredient is that of a first order reaction.

(5) The essential choline needed to produce a given growth is less in birds whose supply has been more drastically curtailed in the pretest period. In such birds, choline chloride in excess of about 0.1%, particularly in the presence of betaine, depresses growth.

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Spectrophotometric Evidence for the Presence of a Leuco Precursor of Both Anthoxanthin and Anthocyan Pigments in Asiatic Cotton Flowers

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INTRODUCTION

During the present century considerable knowledge has been accumulated concerning anthoxanthins and anthocyanins, two of the chief groups of naturally occurring sap-soluble pigments in plants.

Thanks to the researches of Perkin, Wheldale, Robinson, and many others, a large number of these pigments have been isolated, synthesized independently *in vitro*, and structurally identified. The close similarity in structure of the two classes of pigment has always suggested that their natural syntheses in the plant are interrelated. When Willstätter and Mallison (1) and Everest (2) succeeded in reducing the anthoxanthin, *quercetin*, to its analogous anthocyan pigment, *cyanidin*, *in vitro* it was thought that the anthoxanthins were probably the natural precursors of anthocyanins in plant tissues. However, until recently (3) there has been no critical evidence that the conversion anthoxanthin → anthocyanin can actually take place in the plant, and the view more commonly held is that the two classes of pigment are synthesized in parallel from a common precursor. Evidence has been produced that this common precursor is present in rather limited quantities, so that the absolute amounts of anthocyanin and anthoxanthin occurring in flowers tend to be negatively correlated. Furthermore, from theoretical considerations it has been deduced that several independent steps are involved in the conversion of the precursor to either an anthocyan or anthoxanthin pigment (4, 5).

In problems of this nature the geneticist can provide much pertinent information, since his "mutants," in which flower pigmentation is suppressed or reduced as compared with that of "normal" stocks, represent the effect of "blocks" in the chain of gene-controlled enzymatic processes leading to full pigmentation. In fact, mutant types which lack anthoxanthin or anthocyan pigments are likely to be of more interest than types which represent variants in the specific pigments present, although it is the latter which have thus far received most attention.

In Asiatic cotton flowers (*Gossypium* spp.) it has recently been found (3) that the typical "red spot" at the base of the petal is due to the presence of a glycoside of the anthocyan pigment, cyanidin. In addition, 3 different mutant types, "Ghost Spot," "Spotless" and "Basic Spotless," are known, all of which lack the ability to synthe-

size cyanidin. *In combination,* however, two of the mutants, "Ghost Spot" and "Spotless," are able to bring about this synthesis. Genetic, developmental, and chemical evidence were found to agree in the following interpretation:

(a) All 4 flower types, prior to flower opening, contain a leuco substance which can. be extracted from the flower buds and reduced with nascent hydrogen to a magenta pigment resembling cyanidin.

(b) In Basic Spotless types all, or nearly all, of this leuco substance is oxidized to an anthoxanthin pigment, presumably quercetin or its glycosides, which have been found in all cotton flowers examined (6, 7, 8, 9, 10).

(c) Ghost Spot types carry a gene (*G*) in whose presence the anthoxanthin pigment present at the base of the petal is reconverted (reduced) into a leuco substance. In the developing petals this change can be observed as a disappearance of the yellow (anthoxanthin) pigment at the base of the petal, and the accumulation of a leuco substance in the resulting white or "ghost" spot (3). There is reason to believe (see p. 455) that the leuco substance present in mature Ghost Spot petals is identical with the leuco substance present in *young buds* of all 4 flower types.

(d) Red Spot types carry *G*, and, in addition, another gene, *S*, which further reduces the leuco substance produced by *G* to cyanidin.

(e) Spotless types lack *G*, but carry *S*. Since, at a stage equivalent to that at which a spot appears in the petals of Ghost Spot and Red Spot types, they contain no available leuco substance upon which *S* can act, these types have no red spot and are indistinguishable phenotypically from Basic Spotless types.

According to Everest (2) the reduction of quercetin to cyanidin *in vitro* takes place *via* an intermediate colorless substance in which—CH(OH) replaces—CO at position 4 in the pyrone ring of the quercetin nucleus. Robinson and Robinson (11) investigated several leuco anthocyanins which were convertible to corresponding anthocyanidins on boiling with mineral acids. They suggested that leuco anthocyanins contain the groups—CH(OH)·CH(OH)—at positions 3 and 4 in the pyran ring, and that they are converted to anthocyanidins by dehydration. The leuco substance in cotton flowers, like a leuco anthocyanin, is converted to an anthocyanidin on boiling with mineral acids; but since it has been found (3) that the reaction is reversed by milk oxidizing agents, it seems likely that reduction rather than dehydration is involved.

In this paper spectrophotometric evidence is presented which supports this view, and indicates ·that the leuco substance is probably an intermediate product in the *natural* reduction of quercetin to cyanidin, and identical with the intermediate substance produced by reduction of quercetin *in vitro*.

EXPERIMENTAL

The Relation Between the Naturally-Occurring Leuco Substance and the Naturally-Occurring Anthocyanin

Confirmation by spectrophotometric methods was required on two points, (a) that the anthocyan pigment obtained by *in vitro* reduction

of the naturally occurring leuco substance in Ghost Spot petals is identical with, or very similar to, the naturally-occurring anthocyan pigment in Red Spot petals, and (b) that the naturally-occurring anthocyan pigment is, in fact, a glycoside of cyanidin as indicated by the earlier chemical evidence (3).

Extracts of Ghost Spot and Red Spot petal types (both occurring in nearly related strains of *Gossypium arboreum*) were obtained by macerating fresh petals in ethyl acetate and exhaustively extracting them in this solvent until all anthoxanthins had been removed. The residue (containing the leuco substance in the case of Ghost Spot, and the anthocyanin in the case of Red Spot petals) was next extracted with 3% hydrochloric acid in 95% ethyl alcohol and reduced with granulated zinc. Under this treatment, the extract containing the leuco substance was partially reduced to an anthocyan pigment, while the extract containing the natural anthocyan pigment was apparently unchanged. The extracts were filtered and compared spectrophotometrically.
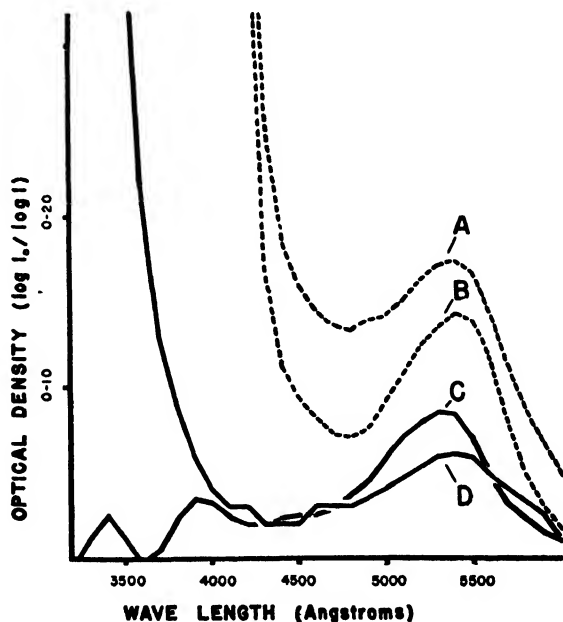
FIG. 1. Spectral absorption of (A) reduced glycosides of quercetin from Upland cotton flowers, (B) reduced quercetin (Eastman), (C) the natural anthocyan pigment from Asiatic Red Spot flowers, (D) the reduced natural leuco substance from Asiatic Ghost Spot flowers. Solvent = 3% hydrochloric acid in 95% ethyl alcohol. The absorption at each wave length in Angstrom units is plotted as optical density, *i.e.*, the logarithmic ratio of the transmission of the solvent (I_o) to the transmission of solute + solvent (I).

To confirm the fact that the natural anthocyan pigment and the pigment obtained by reducing the natural leuco substance were identical with cyanidin, the extracts were also compared spectrophotometrically with two known sources of the latter pigment in the same solvent. These were obtained by reduction of (a) a sample of quercetin obtained from Eastman Research Laboratories, and (b) the natural anth-oxanthin pigments of Upland cotton flowers (*G. hirsutum*) which are known (9) to consist entirely of glycosides of quercetin. The absorption curves obtained for all 4 samples, natural anthocyanin, reduced leuco substance, reduced quercetin, and reduced Upland anthoxanthin are presented in Fig. 1.

Data given by Sando and Bartlett (12) show that quercetin and its glycoside, isoquercitrin, have closely similar spectra with two maximal absorption bands, the band corresponding with the longer wave length being located between 3500 and 4000 Å (see also Fig. 3). Bearing this in mind in considering the data in Fig. 1, it is clear that the reduction of quercetin and glycosides of quercetin was not complete since there is a maximal absorption region at or below 4000 Å. However, in the case of both, a new band has been developed as a result of reduction with a maximal absorption at 5400 Å which must correspond to cyanidin.
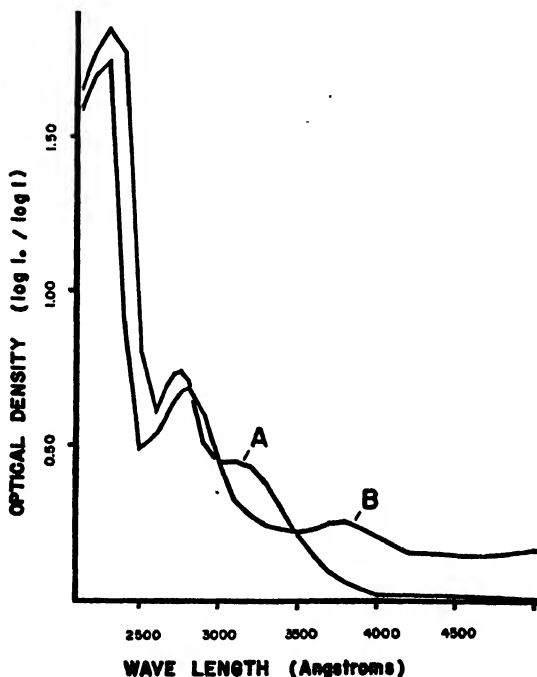
FIG. 2. Spectral absorption of (A) the natural leuco substance from Asiatic Ghost Spot flowers, (B) reduced natural anthoxanthins from Asiatic yellow petaled flowers. Solvent = aqueous 3% hydrochloric acid.

Comparison of the absorption curve of the reduced leuco substance in Fig. 1 with that of the unreduced leuco substance shown in Fig. 2 shows that here, too, reduction was not complete, since both show maximal absorption below 3500 Å. Nevertheless, reduction has resulted in the production of a new band at 5400 Å, i.e., identical in position with that of cyanidin produced by reduction of quercetin and glycosides of quercetin.

The natural anthocyanin shows little absorption in the near ultraviolet region, but has a maximal absorption at 5300 Å, i.e., in a similar location to, but not identical with, the corresponding peaks of the other 3 curves shown in the text figure. This slight discrepancy may be due to the small pH differences. Further investigation was not carried out as it was found that in neutral solution both the reduced leuco substance and the natural anthocyan pigment gave the blue coloration with ferric chloride which is a specific test for cyanidin.

The Relation Between Naturally-Occurring Anthoxanthins and the Naturally-Occurring Leuco Substance

Perkin (6, 7) concluded that yellow flowered Asiatic cottons, carrying the gene Y_a (13), contain two anthoxanthin pigments: *gossypitrin* (a glycoside of *gossypetin*) and *isoquercitrin* (a glycoside of *quercetin*). Later, Neelakantam et al. (10) found gossypetin, gossypitrin, quercetin, and *herbacitrin* (a glycoside of *herbacetin*) in flowers of the same genetic type. Notwithstanding these discrepancies, examination of the available literature shows that two generalizations appear to be valid; (a) that yellow flowers, whatever the species, always contain gossypetin or its glycosides, while non-yellow flowers do not, and (b) that all cotton flowers, whatever their color, contain quercetin and/or its glycosides. It would seem, therefore, that quercetin is the primary anthoxanthin in the cotton flower and that the yellow flowered types, carrying the gene Y_a, are able to further oxidize quercetin to gossypetin. No explanation can as yet be offered for the occurrence of herbacetin, which is apparently only found in the Asiatic species.

Preliminary experiments showed that the anthoxanthins extracted from yellow flowered Asiatic types (*G. arboreum*) gave no (or only faint traces of) anthocyan pigment when reduced with nascent hydrogen in alcoholic solution, thus differing sharply from samples of quercetin (Eastman), which are readily reduced to cyanidin under similar conditions. By using a different method, however, it was found that both quercetin and the natural anthoxanthins from yellow flowers could be converted into an anthocyan pigment in two stages. The pigments were reduced in *aqueous* solution with zinc and 3% hydrochloric acid. Unreduced anthoxanthin was removed by shaking with ethyl acetate, and the resulting colorless solution yielded cyanidin on boiling with strong hydrochloric acid.

These preliminary investigations provided good chemical evidence that quercetin, and possibly more highly oxidized anthoxanthins also, can be reduced to cyanidin *via* an intermediate leuco-substance. The similarity in structure of quercetin and cyanidin does not admit the
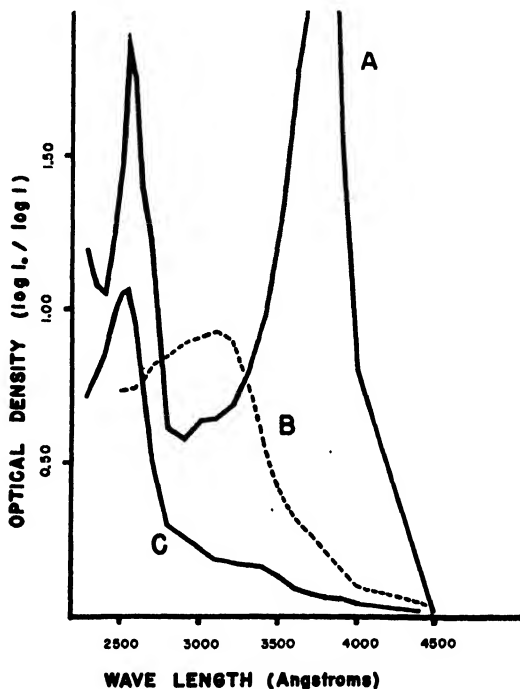
FIG. 3. Spectral absorption of (A) quercetin (Eastman), (B) impurity from the natural leuco substance, (C) oxidation product of the natural leuco substance. Solvent (A and C) = 3% hydrochloric acid in 95% ethyl alcohol, (B) isoamyl alcohol.

possibility of many alternative intermediates, which suggested that the naturally-occurring leuco substance and the intermediate colorless substance obtained *in vitro* by the reduction either of quercetin, or of the natural anthoxanthins from yellow flowered Asiatic types, are identical. This was confirmed spectrophotometrically. In Fig. 2 the spectral absorptions of reduced natural anthoxanthin and natural leuco substance, both in aqueous 3% hydrochloric acid, are compared. The positions of the two main bands correspond almost exactly at 2300 Å and 2750–2800 Å respectively. In addition, there are two minor peaks, at 3750 Å in the case of the reduced anthoxanthin, and at 3100

Å in the case of the natural leuco substance. The band at 3750 Å is identical with one of the quercetin bands (Fig. 3), and may be safely attributed to incomplete reduction. The band at 3100 Å represents an unknown impurity which was later separated from the leuco substance by shaking with isoamyl alcohol, in which the leuco substance is practically insoluble. In isoamyl alcohol the impurity has a single absorption band at 3100 Å (Fig. 3), and has none of the properties of an anthoxanthin or leuco substance. Both solutions, whose absorptions are shown in Fig. 2, yielded cyanidin on boiling with hydrochloric acid.

It is of interest to note that no success was obtained in preparing the leuco substance by reduction of the quercetin glycosides (isoquercitrin and quercimeritrin) which occur in the flowers of Upland cotton (*G. hirsutum*). Unlike the anthoxanthins from yellow Asiatic flowers and quercetin itself, these glycosides were readily reduced in *aqueous* solution to cyanidin, and it was not found possible to identify spectrophotometrically any intermediate product. No explanation can be offered for this difference in ease of reduction between quercetin and its glycosides.

Attempts to convert the natural leuco substance to quercetin by the action of oxidizing agents were unsuccessful. In alcoholic solution, the leuco substance yielded a pale yellow oxidation product on boiling with 3% hydrogen peroxide. This substance had some of the properties of an anthoxanthin, as its color deepened in alkaline solution and it gave an orange precipitate with basic lead acetate. It differed from an anthoxanthin in being only slightly soluble in ethyl acetate and in giving a negative reaction with ferric chloride. Its spectral absorption is compared with that of quercetin in Fig. 3. It can be seen that the oxidation product absorbs strongly in the near ultraviolet region, with a maximum at 2550 Å, identical with the lower wave length maximum of quercetin in the same solvent. Unlike quercetin, it has no corresponding peak at 3750 Å.

In spite of the failure to oxidize the leuco substance to quercetin *in vitro*, there is some evidence that this reaction may be possible under suitable conditions. Ghost Spot petals were exhaustively extracted first with ethyl acetate and secondly with isoamyl alcohol, the residue then being extracted with hot ethyl alcohol. The solution so obtained gave negative tests for the presence of anthoxanthin. It was then evaporated to dryness, and in the process it was noticed that a yellow color developed with increasing concentration. Part of the residue was soluble in ethyl acetate giving a pale yellow solution, turning pink on reduction with nascent hydrogen. It seems likely that a partial oxidation of the leuco substance had occurred in boiling, with the production of a small amount of quercetin. Unfortunately sufficient material was not available to check these results on a larger scale, and they cannot therefore be regarded as conclusive. However, Everest's postulated structure of the leuco substance

(see introduction of this paper) suggests that conversion to quercetin would require mild oxidation with a simultaneous loss of water at position 4 in the pyrone ring, *i.e.*, under conditions that should be satisfied by the procedure described above.

Considering the spectrophotometric evidence as a whole, it seems clear that the natural leuco substance in Ghost Spot petals is an intermediate product in the reduction of quercetin to cyanidin in the tissues of the flower. But, as mentioned earlier in this paper, the leuco substance is not only found in mature Ghost Spot petals; it occurs in *all* young cotton flower buds, irrespective of their genetic type, before either anthoxanthins or anthocyanins can be detected. In all, except Ghost Spot types, the leuco substance disappears as the flower buds mature, which provides strong presumptive evidence that the leuco substance is the common precursor of both classes of pigment, *i.e.*, capable of being oxidized to quercetin or, alternatively, reduced to cyanidin, according to the genotype of the flower. This does, however, assume that the leuco substances extracted from the different flower buds are identical. Spectrophotometric examination of extracts of the leuco substance from (a) young flower buds of Upland cotton (*G. hirsutum*), (b) young flower buds of Basic Spotless (*G. arboreum*), and (c) mature Ghost Spot flowers (*G. arboreum*), confirmed this assumption, as the 3 extracts gave practically identical absorption curves of the type shown in Fig. 2.

DISCUSSION

The spectrophotometric evidence presented in this paper confirms the genetic, developmental, and chemical evidence reported previously (3), that there is a leuco substance present in cotton flowers which represents an intermediate stage in the reduction of quercetin to cyanidin. The genetic and developmental evidence has shown that this substance may be regarded as a precursor common to both pigments, as it is present in the young flower buds of all cotton flowers before the pigments themselves can be detected. To this extent, the data support the commonly accepted theory that anthoxanthins and anthocyanins are synthesized *in parallel* from a common precursor in the plant. However, the general theory of parallel synthesis supposes that the formation of either anthocyanin or anthoxanthin from the common precursor involves several chemical steps, one involving a ring closure (4). In the cotton flower this is certainly not the case—a

single, gene-controlled chemical step is sufficient to convert the precursor into either pigment. There is good reason to suppose that the situation in *Gossypium* is not unique. A parallel case occurs in *Rudbeckia hirta* (14) in which two mutant types with acyanic flowers, on crossing together, produce a hybrid with cyanic flowers phenotypically indistinguishable from the normal form. Recent studies, which will be published elsewhere, have shown that the central cone of disc florets in the normal type contains cyanidin, while in one of the mutant types ("Black Yellow") the cone contains a leuco substance which is absent from the other mutant ("Red Yellow"). Chemically, the situation seems to be identical with that in the cotton flower, and it would be interesting to know whether a reinvestigation of other, superficially similar cases—*Lathyrus* (15), *Cheiranthus* (16), and *Antirrhinum* (17), for example—would allow of a similar interpretation.

It is clear that, if the conversion of a common precursor into either an anthocyanin or anthoxanthin involves only a single step, then controversies as to whether the two classes of pigment are synthesized sequentially or in parallel lose their significance. One would expect, as one finds in *Gossypium*, that both processes can occur in the same flower. This is most readily illustrated in the case of an Asiatic type known as "Red Margin Spotted," which has a yellow petal with a red margin and also a red spot at the base. In the flower bud, all of the petal except the margin contains anthoxanthin pigment; the margin itself contains anthocyanin. Presumably, at this stage the pigments are synthesized in parallel from the common precursor. At a later stage in development, anthoxanthin at the base of the petal is converted into anthocyanin *via* the precursor, and a basal red spot is produced (3). In the spot area of the petal, pigment synthesis is therefore sequential.

Although somewhat outside the scope of this paper, the data do yield some information on the significance of the glycosidal forms in which the pigments typically occur in the plant. It has been supposed, since the pigments are found as glycosides, that they are probably built up from precursors as such, and that the corresponding hydrolyzed forms (aglycones) rarely occur in the plant. Actually Sando *et al.* (18) have used the fact that quercetin and cyanidin in maize occur as analogous monoglycosides as an argument favoring sequential synthesis of the latter pigment. In *Gossypium* this is certainly not the case; first, because gossypetin and quercetin, in addition to their cor-

responding glycosides, occur in Asiatic cotton flowers (10); secondly, because isomeric glycosides of the same anthoxanthin occur together (8, 9); and thirdly, because the anthocyan pigment is probably combined with a pentose sugar (3), not with glucose, as is the case with the anthoxanthin pigments. The only rational explanation which would fit the observed facts is that the pigments must exist, at least momentarily, as aglycones in the plant tissues, and that the various sugar attachments are under independent genetic control.

SUMMARY

Spectrophotometric evidence is presented which suggests that the leuco substance present in the petals of Asiatic cottons (*Gossypium* spp.) is identical with the colorless intermediate product obtained in the reduction of quercetin to cyanidin *in vitro*. This agrees with genetic and developmental evidence, reported previously (3), that the leuco substance, present in young flower-buds, is a common precursor of both anthoxanthin and anthocyan pigments in the flower petals and convertible to either quercetin or cyanidin by a single, genetically controlled chemical step.

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The Quantitative Requirements of the Rat for Magnesium [1]

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INTRODUCTION

Young rats fed a diet low in magnesium develop a syndrome characterized by vasodilatation, hyperemia, hyperexcitability, and finally death. While some preliminary data are available, the quantitative requirements of the rat for magnesium have not been thoroughly studied. Medes (1) reported that rats fed 6.0 mg. of magnesium in 100 g. of diet containing 0.2–0.4% of calcium made normal growth. Tufts and Greenberg (2) later found that, while 5.0 mg. of magnesium/100 g. of diet containing 0.9% of calcium permitted growth in rats and the production of young, the animals were, however, hyperexcitable and exhibited vasodilatation. The severity of the symptoms were more pronounced when diets containing higher amounts of calcium were fed. Cunningham (3, 4) studied the effect of feeding various magnesium salts on the level of magnesium in blood serum. He reported that with rats the feeding of magnesium carbonate was more effective in increasing the level of serum magnesium than was magnesium sulfate, whereas with sheep the magnesium sulfate was more effective than the carbonate.

The present study was designed to determine the quantitative requirements of the rat for magnesium, using the levels of magnesium in whole blood and growth as criteria and to study the physiological availability of magnesium from various compounds and the young wheat plant. The latter is of interest in connection with "grass tetany" (5, 6) which is characterized by hypomagnesemia. A similar malady has been reported in various parts of the world and is the cause of serious loss, especially in cattle grazed on winter wheat in the High Plains Region of Southwestern United States.

EXPERIMENTAL

Weanling rats of the Sprague-Dawley strain weighing from 45 to 60 g. were used in these studies. The animals were confined to wire bottom cages. Food and distilled

water were supplied *ad libitum*. The percentage composition of the diet was: commercial casein 24, cerelose 64.5, corn oil 4.7, fortified cod liver oil 0.3, and salt mixture 6.5. In preparing the salt mixture (7) the magnesium sulfate was omitted. The following amounts of vitamins in mg. were added to each 100 g. of diet: choline 100, inositol 100, calcium pantothenate 2.0, nicotinic acid 1.0, riboflavin 0.30, thiamin 0.25, and pyridoxine 0.25. The magnesium salts and the finely ground wheat plant replaced an equivalent amount of cerelose in the diet.

After 12 days on the experimental regimens the rats were killed by exsanguination. The magnesium content of the whole blood was determined colorimetrically (8).

Quantitative Requirements

On the basis of information available (2), an exploratory experiment was set up using additions of 0, 5, 10, 15, 20, and 25 mg. of magnesium as the sulfate/100 g. of diet. At the termination of the experiment the blood magnesium values showed a progressive increase from the lowest level in the diet up to 20 mg. of magnesium/100 g. of diet.

On the basis of these observations these experiments were replicated, except that the highest level of magnesium fed was 30 mg. rather than 25. The data on the effect of the level of magnesium as the sulfate in the diet is presented graphically in Fig. 1.
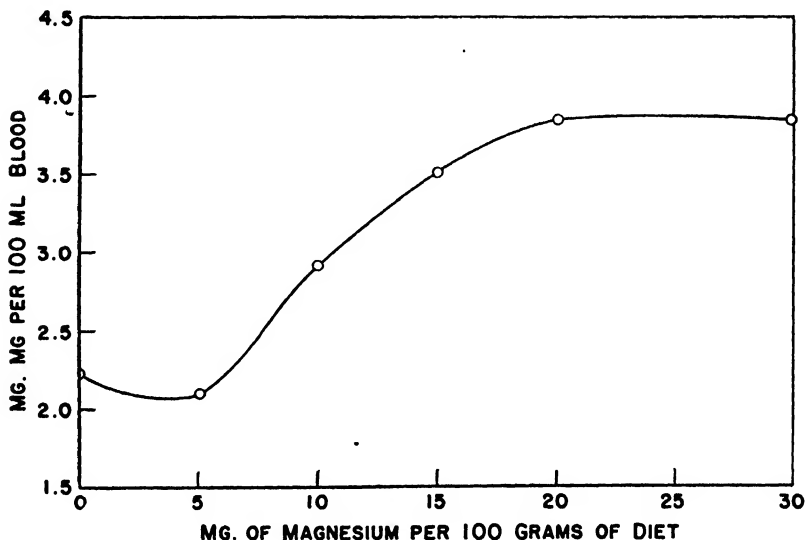
FIG. 1. Effect of feeding different levels of magnesium as the sulfate on the magnesium content of the blood of the rat.

The magnesium content of the blood attained an average value of 3.96 mg./100 ml. on a diet containing 20 mg./100 g. On a diet containing 15 mg. of magnesium/100 g., the average magnesium content of the blood was 3.47 mg./100 ml. On the diet containing 30 mg./100 g. the average magnesium content of the blood was 4.03 mg. which

is not significantly different from the value for rats fed the diet containing 20 mg. of magnesium/100 g. From this it is evident that, on the basis of the magnesium content of the blood, the requirement of the rat was met by the diet containing 20 mg. as the sulfate/100 g. of diet. By actual analysis the basal diet was found to contain less than 1 mg. of magnesium/100 g. The salt mixture used supplied 1.0% of calcium and 0.5% of phosphorus/100 g. of diet.

The average 12-day gain for the rats fed the basal diet was 17.0 g.; for the group fed 5 mg. of magnesium/100 g. of diet, the gain was 21.5 g.; for those fed 10 mg. of magnesium/100 g. of diet, the average gain was 34 g.; and for the 15 and 20 mg. intake, the average gains were 44 and 43.5 g., respectively. Higher intakes of magnesium did not result in more rapid gains. The magnesium requirements for maximum growth agree reasonably well with the requirements as measured by the magnesium content of the blood.

The magnesium content of the blood and growth both appear to be more sensitive measures of the adequacy of the magnesium intake than are hyperexcitability and vasodilatation.

Physiological Availability of Magnesium

The physiological availability of magnesium as the carbonate and as the oxide, and magnesium in the young wheat plant was studied using the magnesium content

TABLE I

Magnesium Content of the Blood of Rats Receiving Various Levels of Magnesium from Different Sources

Source of magnesium	Number of animals	Magnesium added/ 100 g. of diet	Blood magnesium
		mg.	*mg./100 ml.*
MgSO$_4$	8	0	2.3
	11	5	2.1
	12	10	2.8
	12	15	3.5
	14	20	4.0
	8	30	4.0
MgCO$_3$	9	10	2.8
	11	20	3.8
	4	30	4.2
	4	60	4.0
MgO	9	10	2.3
	6	15	2.7
	8	20	3.3
Wheat Plant	3	5	1.9
	7	10	2.5
	7	15	3.0
	4	20	3.6

TABLE II

Physiological Availability of Magnesium

Magnesium source	Level of Mg added to the diet in gm./100 g.			
	10.0	15.0	20.0	Av.
Magnesium sulfate	100[a]	100	100	100
Magnesium carbonate	95	—	95	95
Magnesium oxide	66	57	63	61
Wheat plant	74	73	84	76

[a] Magnesium sulfate was given an arbitrary value of 100, all other values are comparative to the sulfate.

of the blood as the chief criterion. The levels of magnesium added to the basal diet together with other details are shown in Table I. The wheat plant was obtained in its early stages of growth from a field where grass tetany had occurred among cattle grazing on this young wheat. The magnesium content of the wheat plant was 0.18% as determined by the method of Lindner (9).

The magnesium levels of the blood of the rats fed the oxide, carbonate or the wheat plant at a level to supply either 15 or 20 mg./100 g. of diet was not as high as when magnesium sulfate was fed at a corresponding level. The physiological availability of magnesium from the various sources was calculated for each of the suboptimum levels fed. The data obtained with the rats fed graded levels of magnesium sulfate were used as a standard or basis for comparison. The average physiological availability of magnesium as the carbonate and the oxide and magnesium in the wheat plant for the rat were respectively 95, 61 and 76% that of magnesium as the sulfate (Table II).

Since the magnesium from various magnesium compounds and plants is not utilized to the same extent it is apparent that the dietary requirements for magnesium will vary. The requirements of the rat for magnesium as the carbonate is 1.05 times that of magnesium as the sulfate. The corresponding figures for the oxide and wheat plant would be 1.64 and 1.32, respectively. From this it is apparent that the magnesium in the wheat plant is reasonably well utilized.

As might be expected, the symptoms of magnesium deprivation were most severe in rats receiving the lowest levels of magnesium. Mortality resulting from typical convulsions in the groups receiving no added magnesium was high after the tenth day of experiment. When 5 mg. or less magnesium as the sulfate was added to 100 g. of diet, vasodilatation and hyperemia appeared in all animals by the fourth day and disappeared within the next 3 days. When 10 mg./100 g. of diet were fed, these symptoms appeared from the fifth to the ninth day and usually lasted until the termination of the experiment. Some hyperemia was observed in the groups receiving 15 mg. magnesium in 100 g. of diet on the eighth or ninth day, but this seldom lasted for more than 48 hours.

DISCUSSION

Tufts and Greenberg (2) reported that any vasodilatation or hyper-excitability occurring in young rats receiving enough magnesium in

the diet to permit growth appeared during the first 2 or 3 weeks of the experimental period. This indicates that the acuteness of a deficiency was greatest during this period. From the report of Zucker *et al.* (10), it is apparent that the rate of growth of albino rats fed an adequate diet is greatest during the period of 3–6 weeks of age, and declines thereafter. It is to be expected that during this period the magnesium requirement will be the highest and any requirement established during this period would be adequate for the remainder of the growing period. It was during this age period that the experiments reported here were conducted. The length of these experiments, although only 12 days in duration, is believed to be sufficient to reflect the magnesium requirement of the growing rat.

SUMMARY

Using the maintenance of a normal level of magnesium in the blood and growth as criteria, the quantitative requirement of the growing rat for magnesium was found to be approximately 20 mg./100 g. of diet when the magnesium was supplied as the sulfate and when the ration contained 1.0% calcium and 0.5% phosphorus furnished by inorganic salts.

The physiological availabilities for the rat of magnesium as the carbonate, the oxide, and in the wheat plant were calculated to be, respectively, 95, 61, and 76% that of magnesium as the sulfate.

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Isolation and Partial Characterization of Crystalline Tomatine, an Antibiotic Agent from the Tomato Plant [1]

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INTRODUCTION

Previous work (1, 2, 3) has shown that extracts obtained from tomato leaves exhibit antibiotic activity *in vitro* against certain of the fungi and bacteria causing disease in plants and animals. The unknown substances, as a group, responsible for the antibiotic activity of crude tomato leaf extracts has been designated tomatin (2, 3). Because of the potential importance of any good antifungal agent, efforts have been made to isolate and characterize the antibiotic agents present in crude tomato leaf extracts. One crystalline compound possessing antifungal activity but very low antibacterial activity has been isolated from crude tomatin concentrate. The crystalline antifungal agent is designated as *tomatine* to distinguish it from crude or partially purified tomatin referred to in earlier publications (2, 3, 4). It is the purpose of this paper to describe the isolation and properties of crystalline tomatine and to present the evidence which has led to the tentative characterization of tomatine as a glycosidal alkaloid. A detailed chemical comparison of tomatine with alkaloids obtained from other *Solanaceae* plants will be presented in future publications.

Experimental

A crude extract from tomato leaves having an activity of 20 tomatin units (1)/ml. was used as the starting material for the crystallization of tomatine. This concentrate was prepared from dry Red Currant (*Lycopersicon pimpinellifolium*) tomato leaves by 95% ethanol extraction, concentration of the ethanol extract to a thick syrup, extraction of this syrup with boiling water, and concentration of the water extract as described previously (3).

Isolation of Crystalline Tomatine

Ten liters of the crude tomatin concentrate was adjusted to pH 10.0 by adding ammonium hydroxide solution. The precipitate which formed settled rapidly, and the supernatant solution was siphoned off immediately. The precipitate was resuspended in 10 l. of water and dissolved by adjusting the solution to pH 4.0 by adding hydrochloric acid solution. The clear solution (pH 4.0) was adjusted to pH 10.0 by adding ammonium hydroxide solution and the supernatant solution was siphoned off as soon as the precipitate had settled. The precipitate was redissolved at pH 4.0 and reprecipitated at pH 10.0 once more by the same procedure and the precipitate was collected by centrifugation.
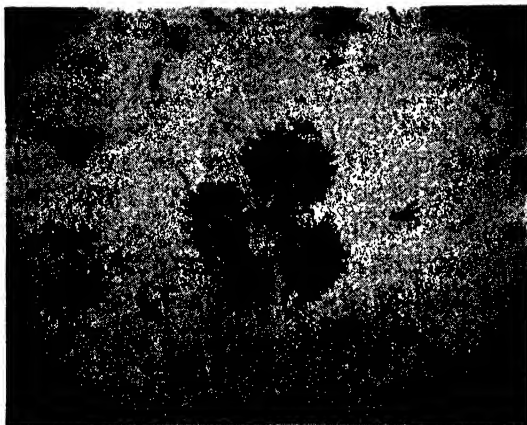
Fig. 1. Crystalline tomatine (from 80% dioxane solution). 100 ×.

The moist alkaline precipitate was dissolved in hot ethanol solution. The alcohol concentration was adjusted to approximately 70% and a final volume of 1.5 l. The 70% ethanol solution of partially purified material (alkaline precipitate) was allowed to stand at 4°C. for 2 days in a refrigerator. During this period an amorphous precipitate formed, which settled and adhered to the bottom of the flask. The supernatant solution was poured off and, upon longer standing in the refrigerator, a second precipitate was deposited. Tomatine was readily crystallized from the first precipitate but not from the second precipitate.

The first precipitate from the 70% ethanol solution was dissolved in hot ethanol and concentrated to dryness at reduced pressure. Hot 1,4-dioxane (peroxide-free) was added to dissolve the residue, and the solution was again concentrated to dryness to remove the remaining ethanol. The residue was then dissolved in 20 ml. of warm 80% dioxane (made alkaline by adding 1 ml. of concentrated ammonium hydroxide/ 100 ml. of 80% dioxane), centrifuged while warm to remove a small amount of gelatinous material, and transferred to a clean flask. Tomatine crystallized in rosettes of short needles (Fig. 1) from this solution on standing overnight at room temperature. Tomatine was recrystallized 5 times from 80% dioxane (ammoniacal), washed with 70% ethanol, absolute ethanol, and finally with diethyl ether. A yield of 2.5 g. of recrystallized tomatine was obtained.

Tomatine, crystallized from dioxane, was also recrystallized from 70% ethanol solution. Tomatine was dissolved in the minimum amount of hot 70% ethanol and then cooled slowly without agitation. (Too rapid cooling of the 70% ethanol solution, or stirring, resulted in the formation of a gel.) Even under the conditions of slow crystallization the crystals are extremely small. Tomatine was recrystallized 3 times from 70% ethanol and dried at room temperature.

Properties

Crystalline tomatine is soluble in ethanol, methanol, dioxane, and propylene glycol. It is almost insoluble in water, ether, and petroleum ether. It is soluble in water, however, as the hydrochloride. Dissolved in 0.1 N hydrochloric acid solution tomatine was found to be levorotatory. Tomatine appears to be stable in strong alkali but is readily hydrolyzed in boiling 1 N hydrochloric acid solution to yield an insoluble crystalline product, *tomatidine hydrochloride*, and a clear supernatant solution rich in reducing sugars. Tomatine gives a positive Molisch test for carbohydrates. The Liebermann test for cholesterol is negative, and the Marquis color test for solanine, as employed by Wolf and Duggar (5), is very faint as compared to solanine. Tomatine, when placed on an electrically heated hot stage preheated to 250°C., melted with decomposition at 263–267°C. The melting point varies, however, depending to some extent upon the rate of heating and the starting temperature. In general, tomatine does not discolor until a temperature of 240°C. is reached. The presence of the carbohydrate moiety in the tomatine molecule possibly accounts for its irregular melting point.

Analysis

Samples of tomatine crystallized from dioxane and from ethanol, were used for analysis. The samples were equilibrated in an atmosphere of water to remove the alcohol, after which it was possible to dry the samples to constant weight. The

chemical analysis of 3 samples gave the following results: carbon: 57.2–57.9, av., 57.5%; hydrogen: 8.15–8.46, av., 8.32%; nitrogen, 1.30–1.42, av., 1.35%. A minimum molecular weight of approximately 1050 for tomatine is indicated by these results.

Tomatidine·HCl

Tomatine refluxed for one-half hour in 1.0 N hydrochloric acid solution yields a crystalline aglycone (tomatidine·HCl) and a supernatant solution rich in reducing sugars. The chemical nature of the aglycone and the sugars is being investigated, and these results will be reported in a later publication. The insoluble tomatidine·HCl was filtered from the hydrochloric acid hydrolyzate and dissolved in hot 70% ethanol solution. Upon cooling, tomatidine·HCl crystallized in long needles (Fig. 2). It was recrystallized 5 times from 70% ethanol
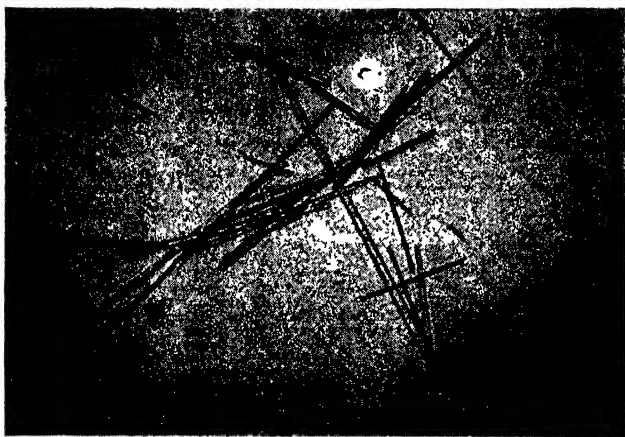
Fig. 2. Crystalline tomatidine·HCl (from 70% ethanol solution). 100 ×.

solution and dried to constant weight for analysis. A Molisch test for carbohydrate was negative. The chemical analysis of two samples gave the following results: carbon: 71.3–71.5, av., 71.4%; hydrogen: 10.43–10.82, av., 10.61%; nitrogen: 3.05–3.11, av., 3.09%; chlorine: 7.78–7.98, av., 7.85%.

Tomatidine

Tomatidine·HCl is readily converted to the free base by dissolving in hot 70% ethanol solution, followed by addition of ammonia. As the solution cools, large flat crystals form (Fig. 3). Tomatidine was further

purified by recrystallization from 70% ethanol. To insure that all of the tomatidine·HCl is converted to the free base, it is advisable to dissolve the dry tomatidine crystals in absolute diethyl ether (tomatidine·HCl is insoluble), then concentrate the ether extract to dryness,
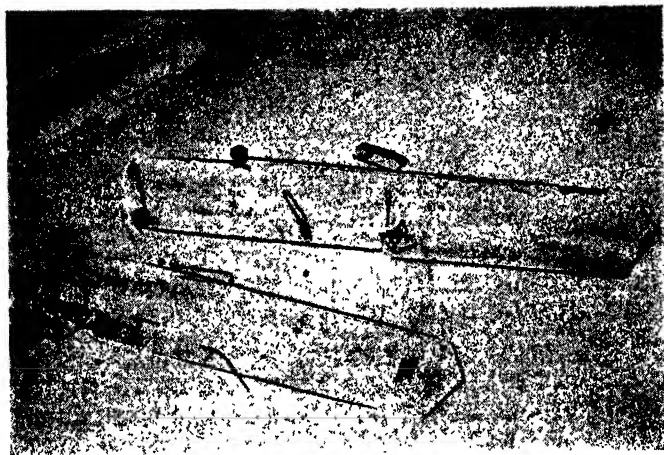
FIG. 3. Crystalline tomatidine (from 70% ethanol solution). 100 ×.

and recrystallize the tomatidine from 70% ethanol. The analysis of two samples gave the following results: carbon: 77.24–77.47, av., 77.32%; hydrogen: 11.04–11.25, av., 11.15%; nitrogen, 3.43 to 3.49, av., 3.46%. A minimum molecular weight of approximately 400 for tomatidine is indicated by these results.

It should be borne in mind that there are some deviations in the analytical results and, for that reason, no empirical formulas have been suggested for tomatine, tomatidine, and tomatidine hydrochloride at present. Part of the difficulty in obtaining consistent analytical values probably can be attributed to small amounts of impurities in the samples analyzed; moreover, these high molecular weight compounds of rather complex structure presented some difficulty in the actual combustion process. From these results, however, it seems possible that tomatine consists of the aglycone fraction, tomatidine, and a tetrasaccharide moiety.

ASSAY

The cylinder-plate assay method, with *Fusarium oxysporum* f. *lycopersici* as the test organism, used previously (1) for measuring the antibiotic activity of crude to-

mato plant extracts and partially purified materials is not applicable for the quantitative assay of crystalline tomatine. Tomatine is only slightly soluble at neutral pH values and would, therefore, be only sparingly soluble in an agar medium at pH 6.6–6.8 as used in the cylinder-plate assay method. Accordingly, only small zones of inhibition could be expected and are obtained with pure tomatine, whereas, with the ' crude material the buffering action of impurities increases the solubility of tomatine and larger inhibition zones are produced. The solubility of tomatine in the nutrient agar medium may be increased by dissolving tomatine in either potassium dihydrogen phosphate or potassium phthalate buffers (pH 4.5). These buffered tomatine solutions assayed in the usual way, using *Fusarium oxysporum f. lycopersici* as the test organism, showed a marked increase in the diameter of the zones of inhibition, whereas, the phosphate and phthalate buffers had no effect.

In an earlier paper (3) it was stated that the alkaline precipitate of the tomato leaf extract accounted for only approximately 15% of the total fungistatic activity of the original extract, while the alkaline supernatant solution (pH 11.0), when readjusted to pH 4.0, showed no fungistatic activity. If the alkaline supernatant solution which has been readjusted to pH 4.0 is used as the solvent and crystalline tomatine is dissolved in it, the apparent fungistatic activity of tomatine is increased approximately 6-fold. This would appear to be similar to the effect obtained when tomatine is dissolved in either phosphate or phthalate solution. At the present time, it appears that the solubility of tomatine in the nutrient agar medium is increased and, therefore, the activity is greater in the presence of impurities present in the crude extract, although a specific effect of these impurities cannot be eliminated at this time.

Antibiotic Spectrum of Crystalline Tomatine

Because of the obvious unreliability of the cylinder-plate method for the assay of crystalline tomatine it has been necessary to utilize an alternative method involving the incorporation of tomatine directly in the nutrient medium.

Tomatine was dissolved in dilute hydrochloric acid solution (0.1 ml. of 0.1 N HCl/10 mg. of tomatine) and then diluted with water to the desired volume. The tomatine solution (pH 3.5) was sterilized and incorporated in various amounts in an agar medium consisting of dextrose, 2.5 g.; yeast extract, 5.0 g.; neo-peptone, 5.0 g.; agar, 15.0 g.; and distilled water to make 1 liter (pH 6.6–6.8). Slants of the various media were inoculated with 12 organisms.

The results (Table I) show that crystalline tomatine is more effective against the pathogenic fungi associated with human disease than

TABLE I

Comparative Study of the Antibiotic Effect of Tomatine and Solanine on Microorganisms

Concentration of tomatine (T) and solanine (S), mg./ml. medium
(The results are expressed as mm. diameter of growth or as plus (+) for growth and minus (−) for no growth)

Microorganism	1.0		0.5		0.25		0.1		0.05		0.01		0.0	
	T	S	T	S	T	S	T	S	T	S	T	S	T	S
Achorion gypseum, ATCC 6286	−	−	−	−	−	3.0	8.0	5.0	9.0	7.0	14.0	15.0	25.5	25.5
Achorion schoenleini, ATCC 4822	−	−	−	−	−	−	−	3.0	8.0	5.0	11.0	9.0	15.0	13.5
Blastomyces dermatitidis, Duke 1035	−	−	−	−	−	−	−	4.5	5.0	6.5	11.0	10.0	18.0	17.0
Epidermophyton floccosum, ATCC 9646	−	−	−	−	−	−	3.0	4.0	4.0	6.0	7.0	11.5	20.0	17.0
Microsporum audouini, E 239	−	−	−	−	−	−	−	4.5	4.5	7.5	9.5	17.0	21.0	21.0
Trichophyton mentagrophytes, ATCC 9533	−	−	−	−	4.5	−	5.0	4.0	6.0	5.5	11.0	12.5	22.0	21.0
Fusarium oxysporum f. *lycopersici*, W R-5-6	14.0	10.0	14.5	12.0	15.0	15.0	15.5	19.0	17.0	21.0	24.0	27.0	32.0	31.0
Penicillium-notatum, NRRL 124B21	8.0	−	8.5	−	9.0	3.0	10.0	10.0	11.0	10.0	13.0	12.5	14.0	14.0
Candida albicans, Duke 1036	−	−	−	+	−	2+	−	3+	+	4+	2+	5+	5+	5+
Candida albicans, E 3147	−	±	−	+	−	2+	±	3+	2+	4+	3+	5+	5+	5+
Escherichia coli, NRRL B210	4+	4+	4+	5+	5+	5+	6+	6+	6+	6+	6+	6+	6+	6+
Staphylococcus aureus, NRRL B313	+	+	2+	2+	3+	3+	3+	3+	3+	3+	3+	3+	3+	3+

against the fungus *Fusarium oxysporum* f. *lycopersici* which causes tomato wilt. Crystalline tomatine, even at 1 mg./ml. concentration, is almost completely without effect on *E. coli* and only slightly effective toward *S. aureus*. It thus appears that crystalline tomatine does not have as much antibacterial action as reported previously for partially purified material (3). The effectiveness of tomatine was increased 4-fold when the first 6 pathogenic fungi listed in Table I were tested in liquid medium. This effect is undoubtedly due to better contact between the organism and tomatine. In subsequent tests, it was found that the antibiotic properties of tomatine were due to the tomatidine portion of its molecule.

The partial antibiotic spectrum of solanine is compared with tomatine in Table I. Although, solanine and tomatine have similar antibiotic properties, they are not identical compounds, nor are their respective aglycones, solanidine and tomatidine, identical, as will be shown in a future publication.

On the premise that the alkaloids present in a plant extract might be responsible in part for its antibiotic activity, approximately 40 different alkaloids and related compounds were tested for antifungal activity toward *Fusarium oxysporum* f. *lycopersici* using the cylinder plant assay technique. Of those tested, only 7 showed definite inhibitory activity, but it should be borne in mind that this assay technique may not be a satisfactory method for screening such compounds. It is of interest to note that alkaloids such as berberine, sparteine, and sanguinarine, and compounds such as atabrine and lepidine (4-methylquinoline), exhibit antifungal properties toward a considerable number of the human pathogenic fungi. These results suggest that a good antifungal agent may be found in a cyclic nitrogen compound containing quinoline or related structures.

SUMMARY

A crystalline compound, tomatine, possessing antifungal activity toward plant and animal pathogenic fungi but very low antibacterial activity has been isolated from the tomato plant. Tomatine has been

characterized as a glycosidal alkaloid. Tomatine is relatively stable in alkaline solution, but is readily hydrolyzed by acids to produce a crystalline aglycone, tomatidine, and a supernatant solution rich in reducing sugars. On the basis of the present analyses, the difference in molecular weight of tomatine and tomatidine suggests that tomatine is composed of the aglycone fraction, tomatidine, and a tetrasaccharide moiety. The antibiotic properties of tomatine are attributed to the tomatidine portion of its molecule. Tomatine and solanine are not identical, although it appears that the alkaloids obtained from the *Solanaceae* plants possess antifungal properties.

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The Beneficial Effects of Biotin on Lactation in the Rat [1]

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INTRODUCTION

The beneficial effects of high levels of synthetic pteroylglutamic acid on lactation in the rat (1) prompted a reexamination of the effects of biotin on lactation. In previous studies by Nelson and Evans (2) little effect was shown by the addition of biotin to a purified diet containing 8 crystalline B vitamins; the weaning weights of the young appeared to be slightly increased, although the weight loss of the lactating mother was slightly greater. Similarly, Vinson and Cerecedo (3) reported that biotin had no effect on lactation in rats, and Richardson and Hogan (4), that the omission of biotin gave inconclusive results. In none of these 3 studies was pteroylglutamic acid present in the diet.

The present study reports the beneficial effects of biotin on lactation in the rat whether in the presence or absence of pteroylglutamic acid. The effects of succinylsulfathiazole and of egg albumin on biotin deficiency during lactation have also been investigated.

EXPERIMENTAL PROCEDURE

The short-term procedure for studying lactation is the same as previously described (1, 2). Adult stock animals of the Long-Evans strain, together with their litters, were placed on the experimental diets at parturition. The litters were limited to 6 young, preferably 3 males and 3 females. All animals, mother and young, were weighed every 5 days and the young were weaned on the 21st day following parturition. Diet was given *ad libitum* and the food intake was measured. The criteria used for lactation

performance with this procedure are: (1) the average weight of the young at weaning; (2) the weight change of the lactating mother during the 21-day period; and (3) the total and differential leucocyte count in the lactating mothers at the end of lactation. These criteria have been discussed previously (1, 2). It may be noted that the percentage of young weaned was eliminated early in our studies as a criterion for lactation performance (2). Rats maintained on the basal purified diet 836[2] containing 8 B vitamins during long-term studies (8-10 months) were equivalent to stock rats in this respect (8), *i.e.*, weaned 90% of their young. At least 2 series of litters (each series consists of 10-12 litters) have been used for each experimental group or subgroup in this study, which has been carried out over a period of 2 years.

The basal purified diet 836[2] containing 8 crystalline B vitamins was used throughout this study. Crystalline *d*-biotin and synthetic pteroylglutamic acid were incorporated in the diet as indicated. Succinylsulfathiazole (SST) was used at a 1% level and replaced an equivalent amount of sucrose for 2 experimental groups. In 2 other groups, purified casein was replaced by powdered egg albumin at the same level (24%). Controls were maintained on the natural food stock diet I (8) plus lettuce *ad libitum.*

Total and differential blood counts were carried out by standard procedures at the time of weaning (Day 21). Bureau of Standards certified pipettes, Trenner or Thoma type, were used. Two to three hundred cells, usually the latter number, were counted on the Wright-stained slide for the differential count. blood samples were taken at a standard time (2 P.M. ± 30 mins.).

RESULTS

The data on lactation and maternal leucocytes at weaning are presented in Tables I and II. Groups 1 and 2 (Table I) show that the addition of biotin to the basal diet containing 8 crystalline B vitamins (*i.e.*, absence of pteroylglutamic acid) resulted only in slight improvement in the weaning weights of the young, namely from 40 g. (females) and 42 g. (males) to 43 g. for females and 44 g. for males. Although this improvement is small, it occurred consistently in every series of litters observed. On the other hand, there was no significant change in the weight loss of the lactating mother or in the maternal leucocytes (Table II), even though there was considerable variation from series

[2] The basal diet 836 has been used for the previous studies on lactation (1, 2). It consists of 24% alcohol-extracted casein, 64% sucrose, 8% hydrogenated vegetable oil (Crisco), and 4% salts No. 4. Crystalline vitamins are added/kg. diet: 5 mg. 2-methyl-1,4-naphthoquinone, 5 mg. thiamine HCl, 5 mg. pyridoxine HCl, 10 mg. riboflavin, 10 mg. *p*-aminobenzoic acid, 20 mg. nicotinic acid or amide, 50 mg. calcium pantothenate, 400 mg. inositol and 1 g. choline chloride. One cc. of a fat-soluble vitamin mixture containing 6 mg. α-tocopherol, 115 chick units vitamin D, 800 U.S.P. units vitamin A, and 650 mg. corn oil (Mazola), was given weekly to each litter.

TABLE I

Effect of Biotin on Lactation

Group	Biotin added/ 100 g. diet	No. of litters	No. of young	Av. wt. young day 1	Av. wt. young day 21	Young weaned day 21	Wt. change of mother during lactation[a]	Av. daily food intake[a]
	γ			g.	g.	per cent	g.	g.
				Basal diet				
1	0	70	220♂ 199♀	6.3 6.0	42 40	95	$-26.0^{(65)}$ $(-76\ \text{to}\ +\ 2)$	$27.8^{(52)}$
2	30	55	163♂ 167♀	6.5 6.2	44 43	97	$-30.3^{(55)}$ $(-66\ \text{to}\ +15)$	$26.3^{(46)}$
				Basal diet + pteroylglutamic acid [b]				
3	0	47	144♂ 138♀	6.3 5.9	46 43	95	$+\ 6.7^{(45)}$ $(-19\ \text{to}\ +43)$	$29.8^{(45)}$
4	30	57	166♂ 176♀	6.4 6.0	50 48	97	$+\ 6.4^{(57)}$ $(-19\ \text{to}\ +43)$	$31.9^{(57)}$
				Basal diet + 1% SST + pteroylglutamic acid				
5	0	32	97♂ 95♀	6.3 5.8	46 43	96	$+\ 3.3^{(31)}$ $(-24\ \text{to}\ +26)$	$30.7^{(31)}$
6	30–60	42	134♂ 118♀	6.5 6.0	50 48	97	$+10.2^{(41)}$ $(-28\ \text{to}\ +37)$	$32.5^{(41)}$
				Egg albumin diet + pteroylglutamic acid				
7	0	21	68♂ 58♀	6.3 6.0	40 39	99	$+\ 8.1^{(21)}$ $(-16\ \text{to}\ +40)$	$28.3^{(21)}$
8	60	24	70♂ 63♀	6.4 5.9	42 40	95	$+\ 6.5^{(24)}$ $(-18\ \text{to}\ +29)$	$27.9^{(24)}$
				Stock diet I + lettuce				
9		70	213♂ 207♀	6.3 6.0	50 48	96	$+19.6^{(64)}$ $(-\ 5\ \text{to}\ +66)$	$36.3^{(43)}$

[a] Superscript numbers in parentheses in this column refer to the number of animals on which the average is based. Only mothers weaning 5–6 young are included in the columns on weight change and food intake.

[b] In Tables I and II, Groups 3 and 4 received two levels of synthetic pteroylglutamic acid (275 γ and 550 γ/100 g. diet); Groups 5, 6, 7, 8 were given only the higher level (550 γ).

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TABLE II

Effect of Biotin on Maternal Leucocytes at Weaning

Group	Biotin added/100 g. diet	No. of blood counts	Total WBC /mm.^{3}	Lymphocytes and monocytes	PMN
	γ				
Basal diet					
1	0	42	4,350 (1,550– 7,500)	3,485 (1,320– 5,560) 82%	865 (0–2,770) 18%
2	30	51	4,755 (1,750– 8,700)	3,810 (1,190– 8,350) 80%	945 (0–2,775) 20%
Basal diet + pteroylglutamic acid					
3	0	45	7,460 (3,350–16,600)	5,315 (2,445–11,400) 71%	2,145 (270–5,995) 29%
4	30	55	8,560 (4,550–16,500)	5,955 (2,445–11,400) 69%	2,605 (760–6,300) 31%
Basal diet + 1% SST + pteroylglutamic acid					
5	0	22	7,795 (4,500–10,850)	5,205 (2,800– 8,245) 66%	2,590 (1,490–4,025) 34%
6	30–60	38	8,010 (3,850–16,200)	5,685 (2,710–13,445) 70%	2,325 (525–5,680) 30%
Egg albumin diet + pteroylglutamic acid					
7	0	20	6,360 (2,700–10,050)	4,245 (1,620– 6,835) 65%	2,115 (1,080–3,215) 35%
8	60	24	• 8,015 (4,850–11,350)	5,080 (2,390– 8,510) 63%	2,935 (1,385–3,620) 37%
Stock diet I + lettuce					
9		43	10,850 (6,550–17,300)	7,675 (3,700–14,530) 70%	3,175 (1,260–6,700) 30%

to series. The food intake during lactation was not increased by the addition of biotin.

When the lactation-induced deficiency of pteroylglutamic acid is corrected (compare Groups 1 and 3), the addition of biotin markedly improves the weaning weights of the young from average values of 44 g. (females) and 46 g. (males) up to 48 g., for females and 50 g. for males (compare Groups 3 and 4). Again there is no effect on the weight gain of the mother despite an increase in the daily food intake from 30 to 32 g. However, there is a slight increase of doubtful significance in the number of circulating leucocytes (Table II), *i.e.*, from 7460 cells up to 8560 cells. This increase is evenly distributed between granulocytes and non-granulocytes so that no change in the percentage of cell types results. Two levels of pteroylglutamic acid, 275 and 550 $\gamma/100$ g. diet, were used for both Groups 3 and 4. The lower level (275 γ), which had previously given maximal results in the absence of biotin (1), was found to do likewise in the presence of biotin. It may be mentioned that the variation in the average values (except for weaning weights of the young) from series to series was much more marked in the case of the biotin-supplemented groups than in those not supplemented, *e.g.*, the weight change of the lactating mothers varied from -2.2 g. for one series on the high level of pteroylglutamic acid to $+12.4$ g. for one series on the lower level of pteroylglutamic acid.

.The data for these 4 groups show that the stress of lactation has produced a mild biotin deficiency in the rat despite the absence of avidin or a sulfonamide in the diet. Biotin supplementation under the experimental conditions resulted principally in an improvement in the weaning weights of the young with only slight effects upon the lactating mother. The beneficial effects of biotin were more marked in the presence than in the absence of pteroylglutamic acid.

Effect of Succinylsulfathiazole

The addition of 1% succinylsulfathiazole (SST) to repress the intestinal synthesis of biotin has practically no effects on the biotin deficiency induced by lactation, as can be seen by comparing Groups 3 and 5. The weaning weights of the young, the weight gain of the mother, the average ·food intake, and the maternal leucocytes, are practically identical for both groups. The addition of biotin to the SST-containing diet resulted in the same improvement in the weaning

weights of the young and the weight gain of the mother as in the absence of SST (compare Groups 4 and 6). The food intake was increased to the same extent while the maternal leucocytes were almost up to the same level. The biotin level was doubled (30 γ to 60 γ/100 g. diet) for half of the litters receiving the SST-containing diet but no further improvement resulted.

As previously noted (9) the addition of SST has not revealed any additional deficiency, although lactation on the purified diet supplemented with 10 crystalline B vitamins (Groups 4 and 6) is still not equivalent to that produced by the use of a good stock diet (Group 9), when judged by the weight gain of the mother, the average food intake, and the maternal leucocytes at weaning. The maternal leucocytes for rats maintained on the purified diet are below those of Group 9 in regard to the average values for total WBC, for lymphocytes and monocytes, for granulocytes (PMN), and especially in regard to the lower range for total WBC, *i.e.*, 29% of the rats on purified diets have total counts below 6000 cells, whereas the lowest total count for rats on the stock diet is 6550 cells. Only the weaning weights of the young on the purified diet are up to the standard of Group 9.

Effect of Egg Albumin

When egg albumin is substituted for casein in the basal diet containing pteroylglutamic acid, the beneficial effects of biotin are again shown in the slight improvement in the weaning weights of the young, *i.e.*, from 39 g. (females) and 40 g. (males) to 40 g. for females and 42 g. for males, and in the increase in total leucocytes from 6360 cells up to 8015 cells (Groups 7 and 8). Thirty-five per cent of the total WBC counts were below 6000 cells when biotin was absent and only 13% when biotin was present in the diet. There was no significant change in the weight gain of the mother or in the food intake by the addition of biotin.

Lactation performance on the egg albumin diet supplemented with 10 crystalline B vitamins (Group 8) is equivalent to that on the corresponding casein diet (Group 4) only when judged by the maternal weight gain and leucocytes at weaning. The weaning weights of the young on the supplemented egg albumin diet are markedly inferior. A high level of biotin was used with the egg albumin diet, namely, 60 γ/100 g. diet, so that the average biotin intake on this diet was 16–17

γ daily, thus eliminating any possibility of suboptimal biotin supplementation. The marked difference in weaning weights, 40–42 g. (females-males) in comparison with 48–50 g. (females-males), together with the difference in food intake, 27.9 g. *vs.* 31.9 g., indicates that the powdered egg albumin[3] lacks some factor or factors present in the casein used. Preliminary studies indicate that the unknown factor (or factors) is also present in certain liver extracts and additional studies are in progress.

DISCUSSION

The production of a biotin deficiency in the adult rat by means of the short-term lactation procedure, despite the absence of avidin or sulfonamide in the purified diet, emphasizes the marked increase in vitamin requirements for lactation in the rat.

Biotin has recently been shown to be beneficial for lactation in the mouse (6, 7), although the mouse has been reported to require biotin for growth on a highly purified diet (10). Heretofore, biotin deficiency has been produced in the rat (usually weanling rats) only by the use of egg white or avidin (11, 12), a sulfonamide (13, 14) or similar drug such as streptomycin or marfanil (15, 16). It may be noted that the beneficial effects of biotin are much more definite when the diet is fairly complete (Groups 3 and 4) than when an additional deficiency such as pteroylglutamic acid (Groups 1 and 2) or an unknown factor (Groups 7 and 8) is present.

The failure of succinylsulfathiazole (SST) to accentuate the biotin deficiency induced by lactation is in marked contrast to the accentuation of pteroylglutamic acid deficiency by SST under the same conditions (9). This failure is, moreover, surprising in view of the well-known action of SST in producing biotin deficiency in weanling rats (13, 14). Apparently the biotin requirement for lactation is so high that the amount furnished by intestinal synthesis is insignificant during this period, whereas the biotin requirement for the growth of weanling rats is much lower and intestinal synthesis can furnish all or a major part of the requirement. The fact that the intestinal synthesis of biotin was decreased by the presence of SST under our experimental conditions was shown by the condition of the young weaned and maintained after weaning on the biotin-deficient SST-containing diet. Typical signs of biotin deficiency (alopecia, eczematous dermatitis, including

[3] A recent shipment of powdered egg albumin of the same grade and specifications as previously used over a period of two years has resulted in better lactation than reported in this study. Slight variations for different batches of commercial egg albumin (as is the case with commercial casein) should probably be expected.

spectacled eyes, pigmentation and spasticity) appeared in these young between 30 and 40 days of age, *i.e.*, 10–20 days after weaning. The young maintained on the biotin-deficient egg albumin diet likewise showed the signs of biotin deficiency after weaning.

The slight beneficial effects of biotin supplementation for lactation on the egg albumin diet and the failure to obtain normal lactation by such supplementation are in agreement with the work of Kennedy and Palmer (5). These investigators found that supplementing a 30% egg albumin diet with 6 γ biotin daily (but no pteroylglutamic acid) resulted in some improvement but not in adequate lactation. The data reported in this study show that supplementing a 24% egg albumin diet with 16–17 γ biotin daily together with high levels of pteroylglutamic acid (over 150 γ daily) did not result in the production of young of normal weaning weight. The lack of some factor or factors in powdered egg albumin and its presence in the casein used are clearly indicated.

The high biological value of egg albumin (17) eliminates the possibility of an amino acid deficiency unless it can be shown that the amino acid requirements for lactation are different from the requirements for growth and maintenance of weight. However, the fact that liver extracts are beneficial makes an amino acid deficiency appear unlikely. While there are no reports that untreated egg albumin lacks an unknown factor or factors necessary for the rat, several investigators have reported that egg albumin, or egg white treated in various ways, is lacking in some unknown factor(s).

Sprince and Woolley (18) have shown that coagulated and dialyzed egg white is extremely low in strepogenin, a factor present in casein, certain crystalline proteins, and in liver extracts. Furthermore, mice fed a highly purified diet containing such treated egg white as the sole source of nitrogen need strepogenin concentrates (or casein) for rapid growth (19). Scott, Norris and Heuser (20) have shown that egg albumin autoclaved for 3 hours[4] is low in factor S and/or strepogenin and an "animal protein factor," all necessary for the chick. Cary *et al.* (21, 22) have reported that heat-coagulated egg albumin, and casein exhaustively extracted with alcohol (60 hrs.), lack an unidentified factor X necessary for the rat; this factor is present in liver extracts and in crude casein, or casein which has undergone milder treatment with alcohol. In relation to an "animal protein factor" necessary for the rat, the recent work of Zucker and Zucker (23) should be mentioned. These investigators found that rats maintained on a vegetable protein diet need an unidentified factor (named

[4] The substitution of autoclaved egg albumin for untreated egg albumin in our basal diet results in an extremely acute deficiency during lactation (Nelson and Evans, in preparation).

"zoopherin") which seemed to be identical with factor X necessary for the rat and the "cow manure factor" (24) necessary for the chick; moreover, the properties of "zoopherin" were the same as those reported for one of the "animal protein factors" necessary for the chick.

Whether the unknown factor (or factors) lacking in the egg albumin diet is the same as or different from the factor (or factors) still necessary for optimum lactation on the casein diet, has not yet been studied. It was previously reported (1) that the addition of a liver eluate powder, 0.5–1.0% level in the diet, resulted in better lactation than the addition of synthetic pteroylglutamic acid. In this study the addition of both biotin and pteroylglutamic acid has not succeeded in bringing lactation performance up to the level obtained by the use of the liver eluate powder (*i.e.*, maternal weight gain of 16 g. and total leucocytes of approximately 10,000 cells). It may be noted that Bowland *et al.* (25) have recently reported that rats given a purified diet containing 26% alcohol-extracted casein need an additional factor or factors (present in alfalfa) for optimum growth and satisfactory reproduction and lactation.[5]

SUMMARY

The addition of biotin to a basal purified diet supplemented with crystalline B vitamins improves lactation performance in the Long-Evans rat. The beneficial effects are more marked in the presence than in the absence of pteroylglutamic acid and may be noted principally in the improved weaning weights of the young with only slight effects upon the lactating mother. The data show that the stress of lactation has produced a mild biotin deficiency in the rat, despite the absence of avidin or a sulfonamide from the diet.

The addition of 1% succinylsulfathiazole does not accentuate the biotin deficiency already induced by lactation. This is in contrast with the previously shown marked accentuation of pteroylglutamic acid deficiency under the same experimental conditions. No additional deficiencies are revealed by the presence of succinylsulfathiazole in the

[5] There have recently appeared two reports on unknown growth factors that should be mentioned. Shorb (26) has found that untreated egg white contains some B_{12} but has considerably less TJ factor. Novak and Hauge (27) have reported that specially purified casein (ethanol extraction followed by isoelectric precipitation) lacks an unknown factor present in distillers' dried solubles and necessary for growth in the rat.

diet although lactation on the basal diet supplemented with 10 crystalline B vitamins is still not equivalent to that obtained with a good stock diet.

The beneficial effects of biotin on lactation are also shown when egg albumin is substituted for casein in the basal purified diet. However, the weaning weights of the young on the egg albumin diet, supplemented with 10 crystalline B vitamins including high levels of biotin, are markedly inferior to those from the corresponding casein diet. The data indicate that egg albumin, despite its high biological value as a protein, lacks some unknown factor or factors present in the casein used and necessary for lactation in the rat.

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Studies on the Guinea Pig Factor of Wulzen and van Wagtendonk

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INTRODUCTION

Wulzen and Bahrs (1) demonstrated a deficiency in guinea pigs fed a skim milk diet supplemented with minerals and vitamins, which was characterized by a wrist stiffness. The same workers (2) had previously indicated that a milder form of the syndrome could be obtained when the animals were fed a grain diet devoid of fresh greens. van Wagtendonk and Wulzen (3, 4) have reported that the deficiency could be cured and prevented by raw cream, fresh greens, and by fat-soluble concentrates and crystalline steroidal material obtained from natural sources. Until the recent report of Oleson, van Donk, Bernstein, Dorfman and SubbaRow (5) the work of the Oregon group had not been reported as being confirmed, for Homburger and Reed (6) and Kon *et al.* (7) had submitted evidence which cast doubt on the interpretation of the data of Wulzen, van Wagtendonk and co-workers as to the reality of a specific factor.

In connection with a broad program for the study of unidentified nutritional factors, we were led to attempt duplication of the work of Wulzen and van Wagtendonk. Although we obtained evidence for the existence of the syndrome which they had described, the poor condition and high mortality of the guinea pigs fed the skim milk diet led us to investigate the use of semipurified diets similar to the one described by van Wagtendonk (8). Our experience in producing the characteristic syndrome and devising a system by which purified materials could be assayed are described in this report.

EXPERIMENTAL

The stock diet was composed of oatmeal, salt given *ad lib.*, and a daily supplement of *ca.* 200 g. of fresh cabbage. The skim milk diet used in our early work was identical with that described by Wulzen and van Wagtendonk. The semipurified diet which we have adopted as the basal diet for producing the syndrome is given in Table I. It differs from that described by van Wagtendonk (8) in that irradiated yeast and cod

TABLE I

	Parts
Casein (Labco)	15.0
Cornstarch	67.7
Brewers' yeast (Fleischmann's 50B)	10.0
Salts[a]	6.0
Cottonseed oil (containing oil-soluble vitamins)[b]	1.3
Ascorbic acid	60 mg./week

[a] Salt mixture according to Mackenzie, Mackenzie and McCullum, *Biochem. J.* **33**, 935 (1939).

[b] Supplements per 100 g. of diet: Vitamin A—1500 I.U.; α-Tocopherol—4.29 mg.; Vitamin D_3—400 I.U.

liver oil are not used; ascorbic acid is given in weekly doses, and α-tocopherol is included in the diet.

The animals, of random sex, weighing about 300 g. were obtained from a commercial source. They were selected for freedom from wrist-stiffness, since it was evident from the start of our research that an appreciable number of animals in each shipment had stiff wrists when received. To avoid any "food shock," the animals were placed on the stock diet for one week following receipt, and this was followed by a 2-week conditioning period, in which the animals were fed one-half oatmeal and one-half basal diet plus cabbage for one week and then the basal diet plus cabbage for the second week. At the end of this period the animals were placed on the basal diet alone and the test period for the development of wrist-stiffness was begun. Under this regime the animals ate well and the mortality was extremely low in the initial stages of the experiments.

The deficiency was measured according to the method of van Wagtendonk for the degree of flexibility of the wrists, an average being taken of both wrists. In this method 4-degree stiffness is complete freedom from the syndrome or a flexing action of 90°, while 1-degree of stiffness is complete absence of flexing action. A 3-degree (60° flexing) stiffness was generally observed in animals on the basal test diet after 4–6 weeks. This degree was taken as definite evidence of the syndrome of the deficiency, and such animals were used to test the curative agents. It had been found that, if animals more severely deficient were used, the assay of materials was irregular and difficult to quantitate.

In the preventive tests, the length of time a group of animals remained above 3-degree stiffness was taken as an estimation of the effectiveness of the supplement, while in the curative tests the change in the stiffness rating from 3 degrees during two consecutive 5-day periods was measured. The use of the two consecutive 5-day periods enabled one to be surer of the response, since the results at the end of the first 5 days might be questionable. Evidence for a "cure" was assumed to have been obtained if half of the animals showed a change from 3 to 3.5 or 4.0 in the 5- or 10-day test periods. At times, our animals showed an increase in stiffness during these periods, but at no time did we experience spontaneous cures under our conditions of management and with our diet.

RESULTS

Animals which had been properly selected for freedom from wrist-stiffness could be maintained in that condition for months when fed the stock diet (*cf*. Table II). Those animals which had signs of wrist

TABLE II

Effect of Stock Diet Constituents in Preventing Onset of Wrist Stiffness

Diet	No. of animals	Degree of stiffness at various intervals					Survival	
		End of stock period	Start [a]	5th wk.	8th wk.	10th wk.		
Series A								
1. Stock	10	4	4	4	4	4	10	
2. Basal	29	4	3.8	3.0	2.6	1.7	24	
3. Basal+200 g. cabbage	10	4	3.8	3.9	3.6	3.6	8	
4. Basal+10% oats	10	4	3.8	3.2	3.1	2.7	9	
				3rd wk.	6th wk.	12th wk.	17th wk.	Survival
Series B								
1. Stock	10	4	4	—	4	4	4	10
2. Basal	18	3.8	3.3	·--	2.6	1.5	1.3	17
3. Basal+200 g. cabbage	10	3.9	3.5	----	3.7	3.7	3.5	10
4. Basal+10% oatmeal	10	3.9	3.3	3.1[b]	3.5	3.7	3.1	10

[a] Previous to the start of this experiment, the animals of Groups 2, 3, and 4 in both series were on the transition regime of 2 weeks described in the text.

[b] This group continued for first 3 weeks on basal alone. Changed on third week to supplement indicated.

stiffness when they were received showed a varied response when fed the stock diet, which leaves doubt as to the nature of the condition observed. It was for this reason that careful selection of animals was considered imperative to successful assay.

In one experiment, not shown in the tables, 23 pigs with an average stiffness of 1.6 after a long period on the semipurified basal diet were changed to the stock dietary regime. Within 5 weeks this group had changed to an average stiffness of 3.0, with several of the animals

showing complete freedom of the stiffness, and with a loss of only 3 animals in this period. Thus it is possible to show the definite curative effect of the complete stock diet.

Since it was found that the stock diet allowed the animals to remain free of stiffness for great lengths of time, or had curative effects when substituted for the deficient basal diet, it was thought of interest to determine whether the addition of stock diet constitutents to the basal would delay the onset of the wrist stiffness. Data bearing on this question are shown in Table II. They indicate that 10% oatmeal or the full daily supplement of cabbage (*ca.* 200 g. per day) supply enough of the factor to delay greatly the onset of the syndrome. Thus, in series A, the basal diet allowed the animals to show the critical 3-stiffness within 5 weeks, while 10% oatmeal delayed the onset till the eighth week, and cabbage did not allow it to appear before the tenth week, which was the length of time the experiment was carried out. In series B, the same type of result is recorded for a longer experiment. In each case, the stock diet kept the animals free of any symptoms.

These experiments are interesting, since it is estimated that the Ca/P ratios in the diets described in Table II were almost identical, (*ca.*1:1) and the percentage of total P varied between 0.4–0.6%. A consideration of the Ca/P and % P is important in view of the report of Hogan and Regan (9), who showed that on a similar diet the ratio of Ca/P and/or the % P was important in allowing the deposition of calcium phosphate in the soft tissues of guinea pigs. It seems very probable that the wrist stiffness is not dependent on the ration of Ca/P or on the deposition of calcium phosphate in the tissue, since we have found no evidence from X-ray examination of such deposition in animals maintained on the basal diet for 4–6 months, even when they are as stiff as those reported in Table II, series B.

In our earliest work we were able to produce the deficiency on the skim milk diet of van Wagtendonk and to cure the syndrome of stiffness by the administration of testosterone propionate as suggested by van Wagtendonk or by changing to the stock diet. Raw cream from cows on winter rations did not seem to contain appreciable amounts of the factor. Although we have discontinued the skim milk diet in favor of the basal diet shown in Table I, it seems worthy of mention that, within the limited amount of data obtained on the ratio of inorganic P: easily-hydrolyzable P (IP:EHP), in animals on the skim milk

diet we have tended to confirm van Wagtendonk's data. Thus the ratio IP:EHP is significantly greater in liver and kidney of deficient animals than in those maintained on the stock diet and those cured by changing to the stock diet or by feeding testosterone propionate.

Since test animals were available at the time of the report of Oleson *et al.* (5) it was thought interesting to try ergostanyl acetate which their work showed to have highest activity of any pure compounds tested. In addition we tested α-ergostenyl acetate, which had been furnished us by Dr. R. L. Levin of these laboratories, the activity of which was not reported by Oleson *et al.* The data for these two pure compounds are given in Table III.

TABLE III

Effect of Sterol Esters Using Curative Assay Procedure

Supplement	No. of animals	Degree of stiffness			
		Begin-ning	5 days	10 days	15 days[c]
Series A					
100 γ. Ergostanyl acetate[b]	11	3.0	3.3 (6)[a]	3.7 (10)[a]	3.4
100 γ. α-Ergostenyl acetate[b]	8	3.0	3.7 (7)	3.9 (8)	3.2
Series B					
25 γ Ergostanyl acetate	8	3.0	3.0 (1)	3.0 (1)	—
25 γ Ergostenyl acetate	7	3.0	3.1 (3)	2.8 (1)	—
100 γ Ergostenyl acetate	8	3.0	3.4 (4)	3.3 (4)	—

[a] Parentheses indicate number of animals considered to show definite curative response; *i.e.*, 3.0 → 3.5 or 4.0.

[b] Physical constants, Ergostanyl acetate: m.p. 145–146°C.; $[\alpha]_D = +8.0$. α Ergostenyl acetate: m.p. 112–113°C.; $[\alpha]_D = -2.6$.

[c] Five days after discontinuing supplement.

These data indicate that α-ergostenyl acetate may be somewhat more active than ergostanyl acetate, and that the amounts of either required by the animals under our experimental conditions is very much greater than that indicated by the work of Oleson *et al.* on a natural pelleted diet. Other tests have indicated that the crude sterol

fraction obtained from the mussel *Modiolus demissus-Dillwyn* is inactive at a level of 5 mg./day.

As indicated in Table III, it appears that animals which have been "cured" within the 5- or 10-day test period should be placed on a subsequent period without the active supplement to determine whether relapse to a deficient state will ensue. By this means we believe the wrist stiffness assay procedure can be made more reliable. Likewise, animals which have not responded to a supplement within the test period should either be changed to a stock diet or given an effective supplement to be sure they were capable of reacting to a known curative agent. This is necessary because of the fact that, as the animals become older and have gone through several test periods followed by curative periods, an appreciable number seem to become "fast" to a known curative agent. However, contrary to the report of Oleson *et al.* we have found no evidence in our work to believe that spontaneous cures occur with the basal diet described and under our experimental procedure.

DISCUSSION

In general, our work, as well as that of Oleson *et al.*, tends to confirm the findings of Wulzen and van Wagtendonk. However, there are significant differences, which probably are explained more on the differences in diet and management of animals than on any other basis.

It should be pointed out that the diet used by van Wagtendonk, Freed and Ballou (8) contained 10% of irradiated yeast, which, in the case of most of the commercial irradiated yeast products, would have furnished toxic levels of vitamin D to the animals. It is also possible that the commercial pelleted diet used by Oleson *et al.* (5) may have been deficient in vitamin E, for similar diets have been found in this laboratory to require supplementation with α-tocopherol.

Although Oleson *et al.* showed that α-ergostenol at 100 γ/day had curative properties, they did not report having used γ-ergostenyl acetate. Since their data contain a number of facts which are hard to reconcile with the slight variation in structure of the compounds tested, such as the wide variations in the activity of esters of ergostanol, the relative activity of α-ergostenyl acetate and ergostanyl acetate found by us is of considerable interest, especially when it is realized that our

data indicate ergostanyl acetate to be between 1/20 to 1/100 times as active as Oleson *et al.* reported.

A matter which is of considerable importance is the Ca and P content of the diet, since Hogan and Regan have reported calcium phosphate deposition on a basal diet similar to the one used in our work, and since van Wagtendonk and Lamfrom (10) reported finding a similar condition in guinea pigs in an advanced stage of wrist stiffness produced by skim milk diets. It should be noted that, if one calculates the additional P from the yeast and casein in the diet of Hogan and Regan, one finds the P to be 0.5% and 0.9% for their two diets, which compares with 0.58% P of our basal diet and 1.0% P for the skim milk diet of Wulzen and van Wagtendonk. Thus, it appears that the wrist-stiffness can occur on diets of high or low P content, but the calcium phosphate deposition may only be produced easily on the high P diet.

ACKNOWLEDGMENTS

SUMMARY

We have confirmed the occurrence of the guinea pig wrist-stiffness syndrome described by Wulzen and van Wagtendonk and coworkers using a semipurified diet of well-known ingredients, and with Ca and P at levels and in a ratio within the accepted limits for animal rations.

We have described procedures for managing the animals so that the assay of active compounds by the wrist stiffness test is not difficult.

We have confirmed the presence of the antistiffness factor in cabbage and oatmeal, and have shown that the wrist stiffness syndrome develops on diets with 0.58% P or less.

It has been shown that ergostanyl acetate and α-ergostenyl acetate are effective curative agents at levels of about 100 γ under our experimental regime.

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Carbohydrate Utilization by a Strain of
Lactobacillus bulgaricus[1]

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INTRODUCTION

Discovery of a strain of *Lactobacillus bulgaricus* which requires lactose for immediate growth, and which cannot utilize glucose, galactose, or a mixture of the two for this purpose has been recorded (1). The same organism requires oleic or linoleic acid (2) and an unidentified factor present in yeast extract (3) for growth in a medium containing all of the vitamins and amino acids known to be essential for lactic acid bacteria. The unusual requirement for an intact disaccharide for growth, and the implications of such a requirement with respect to the mode of utilization of disaccharides, have prompted a more detailed investigation of the carbohydrate nutrition of this organism. Results of this study are presented below.

EXPERIMENTAL

Basal Medium

The basal medium, which is similar to those previously described (1, 4), was prepared in lots of one liter each, at twice the desired final concentration. One l. of this double strength medium contained 10 g. tryptic casein digest (4); 0.2 g. asparagin; 0.2 g. cystine; 20 mg. each of adenine sulfate, guanine hydrochloride and uracil; 200γ each of pyridoxal hydrochloride, thiamine chloride, and p-aminobenzoic acid; 400γ each of calcium pantothenate, riboflavin, and nicotinic acid; 10γ of pteroyl-glutamic acid; 2γ of biotin; 10 cc. each of salts A and B (5); 12 g. of anhydrous sodium

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acetate; 2.0 g. of Tween 80 [3]; and 2.0 g. of yeast extract (Difco). In some of the work, Tween 40 [3] (2.0 g.) plus oleic acid (20 mg.) was substituted for the Tween 80; either supplement supplies adequate oleic acid, and both seem to be otherwise equivalent in growth-promoting effects (2).

To prepare inoculum medium, 1 mg. of cysteine hydrochloride, 100 mg. of an appropriate sugar (glucose, lactose, or both) and 5 cc. of water were added per 5 cc. of basal medium. This diluted medium was tubed in 10 cc. lots, autoclaved at 15 lbs. for 15 minutes, then refrigerated until used.

Stock Culture and Inoculum

The culture, a typical strain of *Lactobacillus bulgaricus*,[4] had been carried since isolation (over 10 years) by monthly transfer in litmus milk containing calcium carbonate. In this laboratory, the culture has been transferred biweekly in litmus milk. Following transfer, cultures were incubated at 37°C. until coagulation indicated good growth (24–48 hrs.) and were then held in the refrigerator for the remainder of the two week period. To prepare inoculum for subsequent studies a loopful of this culture was transferred to 10 cc. of the inoculum medium. This was incubated for 16–24 hrs. at 37°C., centrifuged, the cells resuspended in 10 cc. of sterile saline, and one drop of this heavy suspension used to inoculate each experimental culture.

Procedure ·

Operational details resembled those previously described (*e.g.*, 2, 5). Carbohydrates or other materials to be tested were dispensed in a series of rimless, uniform 18 × 150 mm. Pyrex test tubes, water added to 5 cc., then 5 cc. of the double-strength basal medium added. Tubes were covered with aluminum caps, autoclaved at 15 lbs. pressure for 6 mins., cooled, inoculated, and incubated at 37°C. for an appropriate period. In some cases, carbohydrate supplements were sterilized separately and added aseptically to the previously autoclaved medium diluted in such a way that the total volume after supplementation was 10 cc. Growth was estimated photometrically directly in selected culture tubes with an Evelyn colorimeter equipped with an adapter for 18 × 150 mm. test tubes.

RESULTS

Since *L. bulgaricus*, like other lactic acid bacteria, does not grow in the basal medium if carbohydrate is omitted, growth or its absence provides a sensitive measure of the ability of the organism to utilize a given carbohydrate as an energy source. The growth response of this

[3] Tween 80 is a polyoxyethylene derivative of sorbitan monooleate, Tween 40 is the corresponding palmitic acid derivative. For a discussion of these products and their use in bacteriological media see references 2, 6, 7, and 8.

[4] We are indebted to Professor W. B. Sarles for this culture and for information concerning its classification.
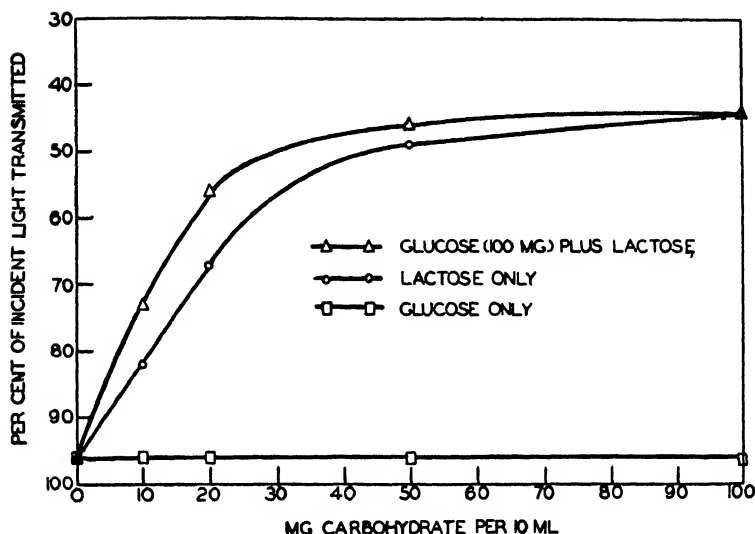
FIG. 1. Growth of *L. bulgaricus* in relation to carbohydrate concentration. Incubation time, 40 hrs.

organism to lactose, glucose, and glucose plus lactose following a 40-hr. incubation period is shown in Fig. 1. Under the conditions of this experiment, glucose alone failed to support growth, whereas lactose was readily utilized. Although not utilized alone, the presence of a constant amount of glucose (100 mg./10 cc.) materially enhanced the growth response to lactose.

TABLE I

Loss of Activity of Lactose for L. bulgaricus on Hydrolysis

Time min.	Hydrolysis[a] (chemical assay) per cent	Apparent destruction microbiological assay[b] per cent
0	0	0
10	14	12
20	28	23
40	54	46
80	82	67

[a] 5% Lactose solution, refluxed with 0.2 *N* HCl. Aliquots were withdrawn for chemical and microbiological assay at the indicated times. Determinations of reducing sugar were made by the Willstaetter-Schudel procedure.

[b] Incubated 26 hrs. The basal medium used in the assay of the various lactose hydrolyzates contained 100 mg. of glucose.

Separate trials showed that, under these conditions, galactose alone, or equimolar mixtures of glucose and galactose were no more effective than glucose in promoting growth of this organism. This result is further emphasized by the data of Table I, which show that hydrolysis of lactose with acid results in loss of its growth-promoting properties for *L. bulgaricus*. The lack of perfect correspondence between degree of hydrolysis and loss in activity is attributed to the enhancing effect of the glucose and galactose formed on growth with lactose, discussed further below. These data seem to eliminate preliminary hydrolytic cleavage of lactose, followed by fermentation of its constituent hexoses, as a mechanism for utilization of this disaccharide.

The enhancing effect of glucose on utilization of lactose was further investigated. Preliminary experiments suggested two possible explanations for the effect, one or both of which might operate: (a) in the presence of lactose, some glucose was utilized, (b) sterilization of glucose with the basal medium might produce decomposition products necessary for, or stimulatory to, growth. That the latter factor does operate is shown by the data of Table II. Unheated glucose, or glucose

TABLE II

Effect of Different Treatments on the Growth-Promoting Properties of Glucose

Lactose,[a] mg./10 cc.	20	20	20	20	0
Glucose, mg./10 cc.	0	3	10	30	30
Treatment of glucose	Per cent incident light transmitted[b]				
Unheated[c]	93	97	93	96	96
Autoclaved separately[d]	93	95	92	97	96
Autoclaved with medium	93	91	87	80	94
Autoclaved with phosphate[e]	93	85	74	65	94

[a] Lactose autoclaved with medium. Each tube inoculated with one drop of heavy suspension; incubated for 22 hrs.

[b] Distilled water = 100, uninoculated medium = 95. Cultures incubated at 37°C. for 24 hrs.

[c] Filtered through "UltraFine" Pyrex bacterial filter, added aseptically to previously autoclaved medium.

[d] 10% Glucose solution, autoclaved at 15 lbs. pressure for 15 mins., diluted, and added aseptically to previously autoclaved medium.

[e] 40 cc. of 10% glucose solution, 200 mg. KH_2PO_4, and 200 mg. K_2HPO_4, autoclaved at 15 lbs. pressure for 15 mins., diluted, and added aseptically to previously autoclaved medium.

autoclaved briefly in distilled water and added aseptically to a medium containing a low level of lactose failed to increase the extremely slight growth obtained with lactose alone. Autoclaving glucose with the medium, or better, the aseptic addition of glucose which had been separately heated with phosphate at pH 7.0, permitted extensive growth of the test organism in the presence of lactose, but not in its absence. Separate experiments showed that these stimulatory substances were also formed when glucose was autoclaved with amino acids (pH 7.0), yeast extract (pH 7.0), or alkali. Thus, decomposition products formed when glucose is autoclaved with the medium, or separately under appropriate conditions, are necessary for rapid growth of this organism, and are undoubtedly partially responsible for the enchanced growth observed in media containing both glucose and lactose (e.g., Fig. 1). Separate experiments have shown that such substances are also produced by heating lactose with the medium, and the low level of lactose used in the above experiment was selected to prevent formation of significant amounts of these stimulatory decomposition products during autoclaving.

Preliminary attempts to determine whether glucose was utilized when lactose was also present were made by comparing acid production in media autoclaved with lactose, glucose, or glucose plus lactose.
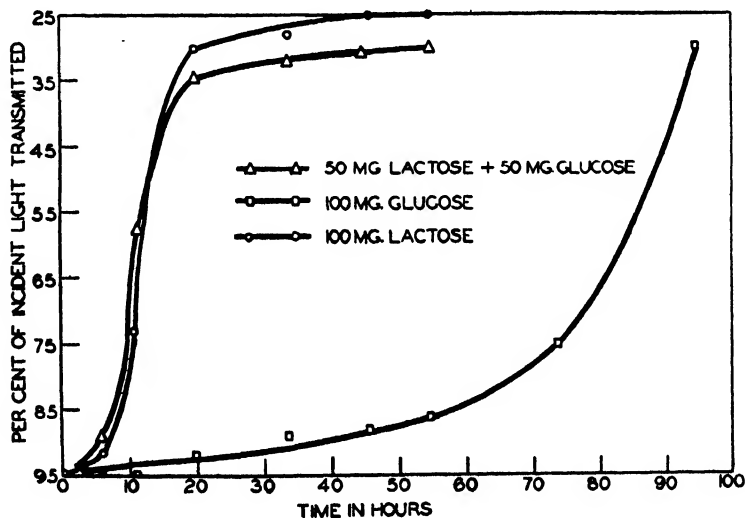
FIG. 2. Effect of time of incubation on sugar utilization by L. bulgaricus.
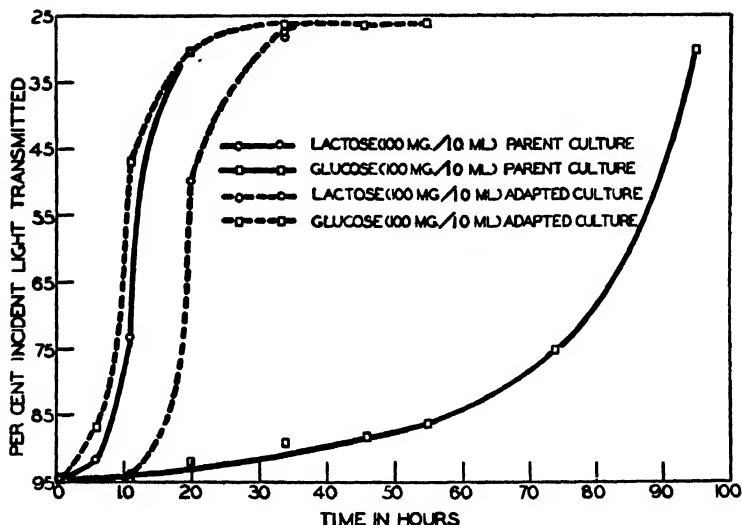
FIG. 3. Comparative growth rates of glucose-adapted and parent strains of *L. bulgaricus* in media containing glucose or lactose.

These were unsuccessful, since on prolonged incubation the organism utilized glucose. This adaptation to utilization of glucose is shown in Fig. 2, where culture turbidity in media containing various carbohydrates is plotted against time of incubation. Growth in lactose-containing media occurs promptly, and reaches maximum levels before significant growth with glucose begins. Eventually, however, rapid development resulting in heavy growth occurs in media containing glucose as the only carbohydrate. Whether ability to utilize glucose in this instance arises by adaptation, selection, or mutation is not known. A transfer from this "adapted" culture developed rapidly in media containing glucose as the sole carbohydrate. Following 26 transfers in this medium, response of the "adapted" culture to glucose and lactose was compared with that of the parent strain, which had been similarly transferred in media containing lactose as the sole carbohydrate. Results are presented in Fig. 3. The "adapted" culture developed as rapidly on glucose as the parent strain did on lactose; it had not, however, lost the ability to grow rapidly on lactose.

The speed with which a given culture acquired the ability to utilize glucose has varied in different experiments from no utilization in 72 hrs., to slight utilization in 40 hrs. and maximum growth in 72 hrs. In

contrast, lactose has always permitted maximum growth in 40 hrs. or less, with slight growth appearing much earlier (see *e.g.*, Figs. 1, 2, 3). Although no detailed investigation has been made, the ability to utilize glucose appears to be acquired more rapidly when heavy inocula are used, when young, actively growing cultures are used as inocula, and when a prolonged autoclaving period is employed with sugar-containing media. Small inocula from older (>24 hrs.) cultures, and short autoclaving periods delay growth on both lactose and glucose, but appear to delay the already slow utilization of glucose proportionately more than that of lactose.

The ability of *L. bulgaricus* to utilize other monosaccharides was next tested. These were tested both in the presence and absence of lactose, and all sugars were autoclaved with the medium. It was thus possible to determine whether the sugar itself was utilized for growth, and whether it enhanced utilization of lactose. Turbidity estimations were made at 24, 48, and 72 hrs., and acid production was measured at

TABLE III

Effect of Monosaccharides on Growth and Utilization of Lactose by L. bulgaricus[a]

Mono-saccharide	Mg./10 cc.	Mg. lactose /10 cc.	Per cent incident light transmitted			cc. 0.1 N acid produced/10 cc. 72 hrs.
			24 hrs.	48 hrs.	72 hrs.	
—	—	0	100	100	100	0.0
—	—	50	100	51	49	4.8
Glucose	100	0	100	80	64	3.8
Glucose	100	50	66	40	40	9.4
Galactose	100	0	100	91	77	3.0
Galactose	100	50	62	37	37	9.8
Mannose	100	0	100	100	99	0.7[b]
Mannose	100	50	67	42	42	7.6
Fructose	100	0	100	63	57	6.6
Fructose	100	50	100	37	36	10.7
Xylose	100	0	100	100	100	0.7[b]
Xylose	100	50	89	49	47	5.2
Arabinose	100	0	100	100	100	0.7[b]
Arabinose	100	50	99	44	41	5.2

[a] Inoculum incubated for 48 hrs. in a medium containing lactose and glucose.

[b] No growth; this amount of acid was produced during autoclaving of the sugar with the medium.

72 hrs. Results are given in Table III. An inoculum culture was used which was 48 hrs. old, and this resulted in delayed initiation of growth (no growth with lactose alone in 24 hrs.). Once initiated, growth was rapid, and achieved approximately maximum levels with lactose alone in 48 hrs., at which time growth with glucose had just begun.

None of the monosaccharides was utilized at 24 hrs. With one exception (fructose) however, each of them increased growth when lactose was also present. For this purpose, glucose, galactose and mannose were equally effective, xylose was less so, and the stimulatory effect of arabinose could be detected only at 48 hrs. At 72 hrs., the test organism grows, albeit not maximally, on glucose, galactose or fructose, and in the absence of lactose. "Adaptation" to growth with these monosaccharides is about equally rapid; in no case is it as rapid as with lactose, where the growth rate is also slowed by the lower sugar concentration. Xylose, arabinose, and mannose were not utilized when present alone. Xylose and arabinose were not utilized even in the presence of lactose; mannose was slowly utilized under these conditions. This is revealed by examination of the data in the last column of the table. The maximum possible acid production from 50 mg. of lactose is approximately 5.8 cc. of 0.1 N lactic acid.[5] Since more than this amount was produced from lactose and mannose, some of the mannose must have been fermented. In the remaining instances, acid production correlates closely with that expected from examination of the growth data. It may thus be concluded that utilization of a sugar and production from it upon autoclaving of the substance speeding growth initiation are independent phenomena.

The availability of various oligosaccharides was next tested, both in the presence and absence of glucose. All sugars were autoclaved with the medium. In the absence of glucose, only lactose was utilized by the organism during the three day incubation period (Table IV). In the presence of glucose, neolactose also permitted extra growth and acid production; other sugars were ineffective. It is significant that the only fermentable oligosaccharides are β-D-galactosides; the failure of the organism to utilize melibiose shows, however, that all β-galactosides are not utilizable.

[5] A quantitative carbon balance run on this organism confirmed the fact, expected from its classification, that only lactic acid was formed from lactose. 96% of the sugar disappearing appeared as *dl*-lactic acid. No detectable carbon dioxide was formed. We are indebted to Mr. W. S. McNutt for these determinations.

TABLE IV

Availability of Various Di- and Trisaccharides to L. bulgaricus[a]

	Growth and acid production	
Sugar	Glucose absent	Glucose present
Lactose	++	+++
Maltose	—	—
Cellobiose	—	—
Melibiose	—	—
Sucrose	—	—
Gentiobiose	—	—
Neolactose[b]	—	—
Raffinose	—	—

[a] — = no growth or no stimulation of growth over that given by glucose alone (Col. 3); ++, +++ = good and heavy growth, or great stimulation of growth over that given by glucose alone (Col. 3). Incubation time, 72 hrs. (Col. 2) or 48 hrs. (Col. 3).

[b] We are indebted to Dr. N. K. Richtmyer for a sample of neolactose [4-(β-D-galactopyranosido)-D-altrose (9)].

Among naturally-occurring carbohydrates, therefore, only lactose supports significant growth of this organism during a 24–48-hr. incubation period. This suggests that the organism might be applicable to the determination of lactose in a mixture of sugars with other biological materials, should such a method be desired.

To test further this configurational specificity of the organism the utility of a number of different β-D-galactopyranosides was tested and compared with that of lactose and a mixture of glucose and galactose (Table V). No other sugar was present in the medium. All β-galactosides tested were utilized; neither of the α-galactosides was utilized. Each of the β-galactosides, like lactose, permitted more rapid growth than did a mixture of glucose and galactose. β-n-Butyl-D-galactoside was a particularly favorable substrate, since the butyl alcohol liberated during its utilization was not deleterious in the concentrations present. In contrast, β-o-cresyl galactoside permitted rapid growth initiation, but the o-cresol formed during the fermentation stopped growth before all of the substrate was utilized. β-Methyl galactoside was the least readily utilized of those tried.

Comparison of relative rates of utilization of the various glycosides is faulty when conducted in the above manner, because the medium contains no source of the stimulatory factors formed by decomposition of sugars during autoclaving, aside from those formed from the glycosides themselves. This is emphasized by data of Table VI. In the absence of glucose, β-methyl-D-galactoside was not utilized in 17 hrs., and not fully utilized after 100 hrs. incubation. In the presence of autoclaved glucose, this galactoside supported very heavy growth in 17 hrs., although autoclaved glucose alone permitted only very slight growth during the same time. Unheated glucose showed no such stimulatory effect on utilization of the galactoside.

An attempt was made to determine the nature of this stimulatory decomposition product of the sugars. A steam distillate of alkali-treated glucose showed no activity; volatile neutral compounds are thus not involved. Data of Table VII show that pyruvic acid is one of the active compounds formed. This compound was considerably more active than autoclaved glucose in stimulating utilization of β-methylgalactoside. Its activity, in contrast to that of glucose, is diminished by heating with the medium. Neither glucose nor pyruvate alone, in the amounts used, permitted growth of the test organism. The magnitude of the growth-promoting effect of pyruvate in this instance is such that it might properly be classed as essential for growth initiation under these conditions.

To obtain the true comparative fermentabilities of various glycosides, therefore, their abilities to support growth or acid production should be tested in a medium which contains pyruvate, or small amounts of an autoclaved reducing sugar. Such a test was carried out, with glucose as the carbohydrate. Turbidity determinations were made at 24 hrs., when no growth had occurred with glucose alone, and before

TABLE V

Availability of Various Glycosides to L. bulgaricus[a]

Compound tested	mg./10 cc.	Per cent incident light transmitted[b]		cc. 0.085 N acid produced/10 cc.
		24 hrs.	88 hrs.	88 hrs.
—	—	100	100	0
Lactose	40	48	45	5.0
Lactose	100	29	26	11.3
β-Phenyl-D-galactoside	40	74	56	3.2
β-Phenyl-D-galactoside	100	29	28	7.9
β-o-Cresyl-D-galactoside	20	81	79	1.2
β-o-Cresyl-D-galactoside	40	83	61	2.2
β-n-Butyl-D-galactoside	40	58	58	3.6
β-n-Butyl-D-galactoside	100	29	22	9.2
β-Methyl-D-galactoside	40	100	71	2.6
β-Methyl-D-galactoside	100	100	25	11.5
α-Phenyl-D-galactoside	40	100	100	0
α-Phenyl-D-galactoside	80	100	100	0
α-Methyl-D-galactoside	50	100	100	0
α-Methyl-D-galactoside	100	100	100	0
Salicin	100	100	100	0
Glucose plus	50	100	29	9.3
galactose	50			

[a] The following individuals kindly supplied samples of pure galactosides: Dr. N. K. Richtmyer (α- and β-phenylgalactosides, α- and β-methylgalactosides); Dr. S. Veibel (β-cresylgalactoside, β-n-butylgalactoside), Dr. S. Roseman, β-methylgalactoside.

[b] Uninoculated medium = 100.

TABLE VI

Effect of Autoclaving Glucose with the Medium on Utilization
of β-Methyl-D-galactoside by L. bulgaricus

Supplement to medium	Amount /10 cc.	Treatment[a]	Per cent incident light transmitted[b]	
			17 hrs.	100 hrs.
	mg.			
None	—	—	93	93
β-Methyl-D-galactoside	100	Autoclaved	93	76
Glucose	100	Unheated	92	45
Glucose	100	Autoclaved	78	37
β-Methyl-D-galactoside + glucose	100 100	Autocalved Unheated	88	43
β-Methyl-D-galactoside + glucose	100 100	Autoclaved Autoclaved	29	24

    a "Unheated" indicates that the supplement was sterilized by filtration and added aseptically to the previously sterilized medium. "Autoclaved" indicates that the supplement was autoclaved with the medium at 120°C. for 6 mins.

    b Distilled water = 100.

TABLE VII

Comparative Activities of Pyruvate and Autoclaved Glucose in Promoting
Utilization of β-Methyl-D-galactoside by L. bulgaricus

Supplement to medium	Mg./10 cc.	Treatment[a]	Galvanometer reading[b]	
			β-galactoside[c] present	β-galactoside absent
None	—	—	78	100
Glucose	1.0	Autoclaved	70	100
Glucose	2.5	Autoclaved	55	100
Glucose	5.0	Autoclaved	47	100
Pyruvic acid	1.0	Unheated	39	100
Pyruvic acid	2.5	Unheated	25	100
Pyruvic acid	1.0	Autoclaved	52	100
Pyruvic acid	2.5	Autoclaved	32	100

    a As in Table VI.

    b Per cent of incident light transmitted; uninoculated medium = 100.

    c 100 mg. of β-methyl-D-galactopyranoside/10 cc. All turbidity measurements were made after 41 hours incubation.

complete utilization of any galactoside had occurred. Under these conditions, the rates of utilization of the various D-galactosides stood in the following order: lactose > β-n-butyl $\gtreqqless$ β-o-cresyl- > β-phenyl- > β-methyl- > neolactose. Again the α-galactosides were inactive.

DISCUSSION

The present study emphasizes two points of interest: (1) existence of organisms which can achieve maximal growth with lactose as an energy source in less time than it requires to initiate growth in glucose, galactose or a mixture of the two, and (2) the necessity of certain decomposition products of sugars, such as pyruvic acid, for rapid growth, even though heavy inocula are used.

In a preliminary publication (1) it was stated that, although certain yeasts were known which ferment lactose more rapidly than equimolecular mixtures of glucose and galactose, this phenomenon had not been observed previously in bacteria. Since then, a paper by Wright (10) has come to our attention, in which a very similar phenomenon was recorded for a strain of *Streptococcus thermophilus*. This organism grew well from small inocula in broth containing sucrose or lactose, but did not do so if glucose, fructose or galactose were used. These latter monosaccharides were utilized for growth and acid production if large inocula were used, but much more slowly than were the disaccharides. The present observations furnish a second example of this same type of phenomenon.

The bearing of these observations on the much-discussed problem of whether disaccharides may be utilized "directly," or whether they are first hydrolyzed to their constituent monosaccharides, which are then utilized, is obvious. A review of the older literature bearing on this subject is given by Wright (10). Since neither glucose, galactose, nor a mixture of the two is fermented or utilized for growth nearly so rapidly as lactose, hydrolysis of lactose to glucose and galactose followed by utilization of these monosaccharides appears excluded.

The recent findings of Doudoroff *et al.* (11, 12, 13) and, of Kagen *et al.* (14), on the reversible phosphorolytic cleavage of sucrose by microorganisms provide a possible explanation for these results. If one assumes that utilization of carbohydrates by lactic acid bacteria proceeds *via* phosphorylated intermediates, it is clear that phosphorolytic cleavage of lactose by a mechanism analogous to that which operates with sucrose (11) would confer an energy advantage on the organism

not present when monosaccharides were utilized. This advantage would be lost if lactose were hydrolyzed before utilization. The same advantage would accrue by phosphorolytic cleavage of β-galactosides other than lactose, and might explain the observation that many synthetic β-galactosides also are utilized much more rapidly than either glucose or galactose. While such a mechanism is attractive, it remains to be proved. Thus, while phosphorolytic cleavage of sucrose by *Pseudomonas saccharophila* does occur, and may explain why cell preparations from this organism oxidize sucrose more rapidly than its constituent monosaccharides, Doudoroff was unable to find evidence for any but a hydrolytic cleavage of melibiose by the same organism, although this sugar, too, was oxidized more rapidly than glucose or galactose (13).

Since glucose is not readily fermented by this strain of *L. bulgaricus*, it would be expected that if phosphorolysis of lactose to glucose and galactose-1-phosphate did occur, and the latter were further fermented, glucose would accumulate in the medium. Preliminary experiments, however, have failed to show accumulation of any free glucose during growth of the organism on lactose. This observation recalls the findings of Morel and Monod (15) with a strain of *Proteus vulgaris*. This organism fermented glucose readily, but did not attack fructose. Sucrose, however, was completely utilized.

The rapid rate of utilization of lactose or other disaccharides, as contrasted with the slow utilization of monosaccharides, may be much more common than the few recorded instances would indicate. The common practices of following fermentation reactions qualitatively rather than quantitatively, and of recording action of an organism on a sugar as plus or minus after extended incubation periods, both tend to obscure this phenomenon, which is of considerable biochemical interest.

That autocalving the carbohydrate with the medium is necessary to permit rapid and consistent growth of many lactic acid bacteria (16, 17 ,18), especially when small inocula are used, has been noted before. Orla-Jensen (16), for example, found that no growth, or delayed growth, of a variety of lactic acid bacteria occurred when glucose was sterilized in distilled water and added aseptically to a yeast-casein medium. Methyl glyoxal, furfuraldehyde, pentoses, or hexoses when autoclaved with the medium permitted rapid utilization of the separately sterilized glucose. Methyl glyoxal and furfuraldehyde were ineffective unless heated with the medium, and the actual stimulatory

substances were not identified. With organisms such as *Streptococcus faecalis*, reducing agents seem to be the sole necessary products formed, and autoclaving can be dispensed with if ascorbic acid, cysteine, yeast extract, or other reducing agents are added to the medium (17). For other organisms, including the strain of *L. bulgaricus* investigated above, additional decomposition products of carbohydrate are required.

The requirement of *L. bulgaricus* for such products can be shown with any of the substrates used as an energy source. It is particularly marked with β-methyl-D-galactoside, which in several experiments remained unfermented for over 80 hrs. in the absence of autoclaved glucose, but which is readily utilized in less than 24 hrs. in its presence. Pyruvic acid was highly effective as a substitute for autoclaved glucose (Table VII), even when added aseptically without heating, and may be the principal active substance formed when sugars are autoclaved with the medium. Orla-Jensen (16, 19) found that acetol and glycollic aldehyde, in addition to methyl glyoxal, were effective stimulants which duplicated the action of autoclaved sugars in promoting growth of several organisms which he studied. These compounds were effective only when autocalved with the nitrogenous components of his medium, and hence were probably not active *per se*. Orla-Jensen does not list pyruvic acid among the active compounds. Smiley *et al.* (18) showed, however, that pyruvic acid or acetaldehyde replaced autoclaved glucose in permitting growth initiation by *Streptococcus salivarius*. Of these compounds, we have not tested acetol and glycollic aldehyde. Methyl glyoxal and acetaldehyde, however, were highly toxic for *L. bulgaricus*. Pyruvate itself, though highly effective at low levels (up to 1 mg./cc.) showed progressively increasing toxicity as the level was further increased.

Smiley *et al.* (18) suggest that compounds such as pyruvate may be required by these organisms as hydrogen acceptors to permit initiation of the dissimilation of carbohydrate. Its action in promoting carbohydrate utilization (and hence growth) by these organisms may be wholly analogous to its effect in eliminating the induction period of glucolysis in yeast or muscle extracts (20).

SUMMARY

The carbohydrate nutrition of a strain of *Lactobacillus bulgaricus* was investigated. For prompt growth, the organism requires lactose:

other disaccharides, including maltose, cellobiose, melibiose, sucrose, gentiobiose and raffinose are not utilized, even following prolonged incubation periods. None of the monosaccharides tested permit prompt growth.

Glucose, galactose, or a mixture of the two, do not support growth during a time interval sufficient for maximum growth with lactose. Hydrolysis, therefore, is probably not the first step in utilization of lactose by this organism. On prolonged incubation, the organism acquires the capacity to utilize glucose, galactose, fructose, and, to a limited extent, mannose. Xylose and arabinose are not fermented. Once "adapted" to glucose utilization, prompt development with either glucose or lactose as energy source occurs.

Like lactose, a number of synthetic β-D-galactopyranosides permit prompt growth. These include β-n-butylgalactoside, β-phenylgalactoside, β-o-cresylgalactoside, and β-methylgalactoside. Neolactose is fermented slowly. α-Galactosides, $e.g.$, α-phenyl- and α-methylgalactosides, are not fermented under any conditions.

For prompt growth with these galactosides or with fermentable carbohydrates, $L.$ $bulgaricus$ requires certain decomposition products derived from sugars by autoclaving with the basal medium, with phosphate, or with other materials. Such products are formed from all sugars tested, including those such as xylose which cannot be fermented. Pyruvic acid has the same action as autoclaved carbohydrates, and hence is probably one of the principal active products formed.

The significance of these various findings is discussed.

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Concerning the Alleged Occurrence of
Hyaluronidase in Skin [1]

A number of papers have been appearing in which the statement is made that skin contains hyaluronidase, and the reference upon which this statement is based is the report of Meyer *et al.* (1). In this paper it was claimed that rabbit skin contains hyaluronidase which, however, requires autolysis for its liberation. This conclusion was drawn from the following observation: A suspension of the skin tissue in $M/15$ phosphate buffer, pH 5.0, was autolyzed at 37°C. for 16 hrs. in the presence of toluene, centrifuged, and the residue extracted with saline solution. The combined saline extract and supernatant was found to have hyaluronidase activity.

Previously, Claude and Duran Reynals (2) had reported that extracts of many organs, including skin, from mammalian species possessed "spreading factor" as demonstrated by the diffusion of India ink in rabbit dermis. No precautions to prevent bacterial contamination were mentioned. Chain and Duthie (3) subsequently compared the ability of mammalian organ extracts to produce the spread in dermis and to reduce the viscosity of hyaluronic acid solutions. Although skin was not studied, it was observed with extracts of other tissues that slight activity could be demonstrated by both methods only when the material was not fresh. The absence of activity in the fresh tissue extract led these authors to conclude that the testes are the only mammalian organs normally containing hyaluronidase.

To determine whether the activity observed by Meyer *et al.* (1) was inherent in the tissue or developed from bacterial contaminants, their experiment was repeated by the present authors. In accord with Meyer and coworkers no activity was found in the fresh skin extract. A portion of the fresh tissue suspension was autolyzed, and another portion was

boiled for 7 mins. to destroy enzymes prior to autolysis. The autolyses were carried out in the presence of a large excess of toluene. A putrid odor was apparent in both the previously boiled and unboiled samples at the end of the autolysis. Both samples were found to contain hyaluronidase in approximately equal concentrations as measured viscosimetrically. The enzyme concentration which developed was sufficient to lower the viscosity of the hyaluronic acid solution to half its initial value in about 10 mins. It is clear that bacterial contamination was the chief, and perhaps only source, of the activity which developed. To show that the enzyme does exist in the skin it would be necessary to demonstrate its activity in sterile samples which were treated under aseptic conditions throughout. The fact that no activity is found in the extract from the ground fresh tissue makes it unlikely that the enzyme is present; and proof that autolysis is required before the enzyme can be demonstrated has yet to be established in a manner in which bacterial contamination is obviated.

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The Occurrence of Zoopherin in Lower Animal Forms

The efforts to conserve animal protein sources in poultry husbandry have not led to success in finding an available plant source which will furnish that unknown ("animal protein factor") which traditionally is obtained from meat scrap, fish meal, *etc*. In rat experiments the essential nature of a water-soluble "animal protein factor," tentatively called zoopherin (1), has also been demonstrated. It is not present in yeast, grains, oil seed meals, alfalfa leaf, dried grasses, or fresh grass (2). Thus far, its presence in fish ("fish solubles") and in meat are known and it can be transferred to the young in milk. Its occurrence elsewhere in nature, especially in lower animal forms, becomes of interest.

The data given below do not lay any claim to being more than approximate. With most of them neither season nor food supply could thus far be experimentally controlled. Oysters were live Long Island oysters bought locally in October. *Busycon* (winkle), *nereis* (sandworm), and *asterias*(starfish) were obtained live from Woods Hole Supply Depot in December. The eggs of the crustacean *artemia salina* were also obtained from Woods Hole. *Lumbricus* (earthworm) was obtained in March from good garden soil which has not been manured for a year. The fruit fly *drosophila melanogaster* (wild type) was grown in the laboratory on the commonly used medium of corn meal, molasses, agar, and yeast, and also on the zoopherin-deficient diet (1) plus agar. For testing purposes the *artemia* eggs were added directly to the diet; the other materials were ground in acetone (usually in a Waring blendor) and brought to dry weight in a current of warm air. The diet to which the test material had been added was fed to the 4-week old offspring from rats on the deficient ration Pr 60 (1). The resulting growth was compared with litter- and sex-matched controls. The negative and positive control received, respectively, the unfortified ration and a ration to which 2% of 1:20 Wilson liver powder had been added. In separate tests the liver powder showed near maximal activity at 0.5% and was maximally active at 1% (1).

Oysters at 0.3% (as well as at the higher levels of 1% and 3%) gave a maximal effect equal to that of liver. *Busycon* at 1% showed distinctly submaximal activity, while at 3% it was maximally active. *Nereis* showed full activity at 3% and 1% but definitely less at 0.3%. In preliminary trials *lumbricus* showed definite activity, although less than *nereis*. Starfish gave little effect at 3% and was not fully active even at

9%. *Artemia* eggs gave partial activity at 0.3% and full activity at 1%. Preliminary trials of *drosophila* at 0.3% showed a definite but submaximal activity.

Intestinal synthesis as the source of the factor in such vegetarian mammalian species as the cow is made highly probable by the work of Bird and associates (3). The domestic fowl, however, seems to require a source in the feed (4). The large amount of insect prey which flycatchers and related species of birds consume is well known. A new significance is given to the persistent feeding of worms to nestling birds and, among adults, the early bird also proverbially catches the first zoopherin meal.

Judging by what has been said about its distribution, zoopherin probably has very little if any significance in plant biochemistry in contrast to most of the known nutritional essentials. It is widely distributed among various animal forms and is essential to the continued existence of at least some species. Hence the proposed name.

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